The Protective Effect of Breast Feeding in Relation to Sudden Infant Death Syndrome

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Declaration

I declare that the work for this thesis was carried out by myself or under my direct supervision.

University of Edinburgh 1999
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Dedication

I would like to dedicate this thesis to my Dad and Mum and to my Fiancé Stuart. Thank you for your love and support in every aspect of my life.

I would also like to remember all the babies who have died from SIDS and their families.

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Abstract

Breast fed infants are at a lower risk of SIDS compared to formula fed infants but the actual protective mechanism of breast feeding in relation to SIDS is not known. A model has been proposed which suggests that SIDS victims die from uncontrolled inflammatory responses to bacterial toxins. Bacteria associated with SIDS include *Staphylococcus aureus* and *Clostridium perfringens*. Both colonise mucosal surfaces and produce superantigenic toxins which induce strong inflammatory responses.

The aim of the study was to investigate the protective effect of breast feeding at two stages of the model: colonisation of mucosal surfaces by toxigenic bacteria; inflammatory responses to toxin.

Experiments were carried out to investigate the effect of breast milk, infant formula and synthetic Lewis antigens on binding of *S. aureus* and *C. perfringens* to epithelial cells. Two different methods were used: the conventional experimental or *in vitro* approach; the second an *in vivo* approach developed to simulate interactions at mucosal surfaces. With the *in vivo* method, breast milk enhanced bacterial binding to cells whilst infant formula inhibited binding; however, for the *in vitro* method, human milk and infant formula enhanced binding due to bacterial aggregation. For both methods, the oligosaccharides Lewis^{a} and Lewis^{b} found in both human milk and infant formula inhibited bacterial binding in a dose dependent manner.

IgA antibodies against the staphylococcal toxins TSST-1 and SEC and *C. perfringens* enterotoxin A (CEA) were present in milk samples from 28 donors.

Most SIDS deaths occur during 2 - 4 months of age when infants have decreasing levels of maternal antibodies to infectious agents. Most deaths occur during the early hours of the morning. Adults are more susceptible to inflammatory responses at night due to lower cortisol levels associated with circadian rhythm patterns. Infants develop these patterns between 7 - 22 weeks, at which time their night-time cortisol levels drop dramatically. Breast fed infants develop these patterns significantly earlier than formula fed infants. Human buffy coats were used to investigate pro-inflammatory cytokine production in response to TSST-1 and to investigate the effect of cortisol levels observed in infants before and after circadian rhythm pattern development. Human buffy coats consistently produced TNF-α and IL-6 but only cortisol levels (>5 $\mu g\ dl^{-1}$) found in infants during the day or at night prior to the change significantly decreased TNF-α and IL-6 production.

The bacterial binding studies indicate protection associated with breast feeding in relation to SIDS could be due partly to enhanced clearance of bacterial aggregates. Human IgA antibodies in breast milk might neutralise bacterial toxins on mucosal surfaces. If the switch to the circadian rhythm pattern occurs in an infant when maternal antibodies are present or an infant is receiving IgA in breast milk, the infant might be able to neutralise a challenge with the toxins; however, if the switch occurs in an infant when antibody levels are low or an infant is formula fed, lower

night-time cortisol levels might be insufficient to control inflammatory responses induced by bacterial toxins alone or in combination with other infections.

Abbreviations

ALTE apparent life threatening event

BI binding index

BSA bovine serum albumin

CEA *Clostridium perfringens* enterotoxin A

CI confidence interval

CMV cytomegalovirus

CSE cigarette smoke extract

D defatted breast milk

DEPC diethyl pyrocarbonate

DMEM Dulbeco's Modified Eagle's Medium

DPT diphtheria-pertussis-tetanus

EBV Epstein-Barr virus

ELISA enzyme linked immunosorbent assay

FCS foetal calf serum

FITC fluorescein isothiocyanate

HRP horse radish peroxidase

IFN-γ interferon gamma

Ig immunoglobulin

IL interleukin

mRNA messenger RNA

MuLv RT murine leukemia virus reverse transcriptase

OD optical density

OPD ortho-phenylenediamine dihydrochloride

OSAS obstructive sleep apnea syndrome

PBS phosphate buffered saline

PCR polymerase chain reaction

RSV respiratory syncytial virus

RT reverse transcriptase

RT-PCR reverse transcriptase polymerase chain reaction

SEC staphylococcal enterotoxin C

SEM standard error of the mean

SIDS sudden infant death syndrome

TNF-α tumour necrosis factor alpha

TSST-1 toxic shock syndrome toxin

v/v volume per volume

w/v weight per volume

W whole breast milk

Chapter One

Introduction

1.1 Sudden and unexpected deaths in infants

Reports of sudden and unexpected deaths in infants can be found in the Old Testament, in Roman records, and in 12th and 13th Century medical and legal literature. These deaths were classified as overlaying. By the late 18th and 19th centuries, the suggested cause of death was fatal interference of heart and lung function by an enlarged thymus [Limerick, 1992].

The first epidemiological patterns of a higher number of deaths occurring during the winter months and early mornings (especially Sunday and Monday), were noted by Wakley in the 19th Century [Limerick, 1992].

1.2 Sudden Infant Death Syndrome (SIDS) - definition and incidence of SIDS

1.2.1 Definition of SIDS

The current definition of Sudden Infant Death Syndrome (SIDS) was made by Beckwith at the 1969 International Conference on the Causes of Sudden Deaths in Infants [Limerick, 1992]. The definition states that SIDS is "the sudden death of any infant or young child which is unexpected by history and in which a thorough post-mortem examination fails to demonstrate an adequate cause of death" [Beckwith, 1970]. Today, a thorough investigation of the scene of the death is strongly recommended.

In 1971, SIDS was accepted as a registrable cause of neonatal death by the Registrar for Births, Deaths and Marriages in Scotland, the Procurators Fiscal and the Coroner's Office [Busuttil *et al.*, 1992].

The use of the term SIDS has increased the thoroughness of the post-mortem examination and stimulated research. Epidemiological studies have been able to identify infants at risk [Busuttil *et al.*, 1992; Limerick, 1992]. One difficulty with SIDS research is the lack of appropriate controls. Ideal controls would be 1 - 6 month old healthy infants who die suddenly due to an accident, but fortunately, these are few in number [Limerick, 1992]. To overcome this problem, many studies include infants who have died of other causes or live infants closely matched for age, sex and socioeconomic background [Blackwell *et al.*, 1995a].

1.2.2 Incidence of SIDS

Although the incidence of SIDS has declined over recent years, it is still the leading cause of postneonatal death in the developed world accounting for approximately 40% of postneonatal mortality [Bernshaw, 1991; Lindsay *et al.*, 1994; Chantler, 1996]. In 1995, the incidence of SIDS in Scotland was 0.7 deaths per 1,000 live births [Brooke *et al.*, 1997].

Ethnic origin influences the incidence of SIDS in a population [Mitchell *et al.*, 1993; Nelson, 1996]. Among white infants in Britain, the incidence of SIDS was reported to be 1.7 per 1,000 live births in the late 1980s. In contrast, Asian infants

during this period had a much lower incidence of SIDS (half or less than half that of white infants), and this is accompanied by lower mortality from respiratory causes [Balarajan *et al.*, 1989; Farooqi *et al.*, 1995]. Many indigenous groups, Australian Aborigines, American Indians and Alaskan natives, have a much higher rate of SIDS. In Australia, among Aboriginal infants the rate of SIDS was 6.1 per 1,000 live births compared to the non-Aboriginal rate of 1.7 per 1,000 live births [Alessandri *et al.*, 1994]. In New Zealand, Maoris had a SIDS rate of 7.4 per 1,000 live births compared to the non-Maori rate of 3.6 per 1,000 live births. This difference between the Maori and non-Maori groups might be due to the fact that the prevalence of risk factors for SIDS including maternal smoking, prone sleeping position and bottle feeding, is higher in the Maori population [Mitchell *et al.*, 1993; Potter *et al.*, 1996].

1.3 A diagnosis of exclusion

SIDS is a diagnosis of exclusion and is made once all alternative causes of death have been ruled out. To diagnose SIDS, a full history, death-scene and thorough post-mortem examination must be carried out. A SIDS death is thought to be due to a final common pathway triggered by several disorders [Berry *et al.*, 1989; Busuttil *et al.*, 1992; Limerick, 1992]. Typical post-mortem findings in SIDS infants are shown in Table 1.1. Hypostatic staining can be used to indicate an infant's sleeping position. No single feature distinguishes SIDS infants from control infants although several differences can be detected by epidemiological studies [Berry *et al.*, 1989; Berry, 1992].

Table 1.1 Typical findings in SIDS infants [Berry *et al.*, 1989]

External

Body apparently well developed and well nourished

Frothy, often blood-tinged fluid around the nose

Cyanosis of lips and nailbeds

Internal

'Large' thymus with petechiae

Petechiae beneath visceral pleura

Epicardial petechiae

Full expansion of lungs

Liquid heart blood

Empty bladder

Prominent lymph nodes and Peyer's patches

Microscopic

Pulmonary congestion and oedema

Mild inflammation of upper respiratory tract

Focal fibrinoid necrosis of vocal cords

Persistent haemopoiesis in the liver

1.4 Developmental and environmental risk factors associated with SIDS

One consistent finding in epidemiological studies is the age distribution of SIDS deaths. SIDS can affect infants aged between one week and one year of life; however, the peak incidence of SIDS occurs during the 2 - 4 month age range and 80% of SIDS deaths occur during the first 6 months of life [Morris *et al.*, 1987; Berry, 1992; Drucker *et al.*, 1992; Lindsay *et al.*, 1994; Blackwell *et al.*, 1995a; Chantler, 1996].

SIDS is associated with a number of developmental and environmental risk factors which are similar to those associated with respiratory tract infections. These risk factors include: the age range affected; winter peak of these deaths in many countries; a mild respiratory or gastrointestinal tract infection prior to death; bottle fed rather than breast fed; exposure to cigarette smoke; the prone sleeping position; no immunisation or late immunisation [Blackwell *et al.*, 1995a; Chantler, 1996].

SIDS is more likely to occur during the winter months and in families of lower socioeconomic conditions (social class IV or V) [Berry *et al.*, 1989; Lindsay *et al.*, 1994; Blair *et al.*, 1996; Chantler, 1996]. Low birthweight infants who are premature ($\leq$ 36 weeks) or who had intra-uterine growth retardation and are small for gestational age or below the given 5th centile, are also at greater risk of dying from SIDS [Berry *et al.*, 1989; Brooke *et al.*, 1997]. Maternal risk factors associated with SIDS include a young and / or unmarried mother, high parity, high birth order, maternal smoking or opiate / barbiturate addiction and a short inter-pregnancy

interval [Berry *et al.*, 1989]. SIDS is more likely to occur during the early hours of the morning (midnight to 6.00 a.m.) and it has been suggested that sleep might be involved in the mechanism of some SIDS deaths [Schechtman *et al.*, 1995; Cornwell *et al.*, 1998].

Male infants have an increased risk of SIDS, 1.84-fold that of females (95% CI 1.22 to 2.77) [Brooke *et al.*, 1997]. One epidemiological study has found a very similar prevalence of SIDS risk factors for both males and females and has suggested that the increased risk associated with male infants is due to an inherent factor [Mitchell *et al.*, 1997].

1.5 Hypotheses developed to explain a SIDS death

A number of different hypotheses have been proposed to explain the mechanism of a SIDS death and are listed in Table 1.2 [Berry *et al.*, 1989]. It is unlikely that one single hypothesis can explain the mechanism of a SIDS death.

Table 1.2 Hypotheses to explain a SIDS death [Berry *et al.*, 1989]

Infanticide and Filicide

Infective
- Overwhelming virus infection
- Septicaemia
- Anaphylaxis secondary to infection
- Infant botulism
- Common toxigenic bacteria in upper respiratory tract infection

Overheating
- Hyperthermia (± infection)
- Malignant hyperpyrexia trait

Hypothermia

Respiratory
- Prolonged sleep apnoea
- Obstruction of upper respiratory tract
- Period of physiological instability of respiratory control
- Apnoea secondary to oesophageal reflux
- Surfactant deficiency

Cardiovascular
- Prolonged Q-T interval
- Abnormal conduction pathways
- Myocarditis

Immunological
- Immunodeficiency
- Anaphylaxis (cows' milk, house dust mites, etc.)

Metabolic
- Hypoglycaemia
- Hypernatraemia
- Inborn errors; Acyl CoA dehydrogenase deficiency

Nutritional
- Hypernatraemia
- Deficiency of trace elements
- Vitamin deficiency

Endocrine
- Elevated tri-iodothyronine
- Absent parathyroids

Toxic
- Common drugs and poisons
- Carbon monoxide
- Lead

Vaccination

1.5.1 SIDS and hypersensitivity to cows' milk

One early hypothesis stated that SIDS occurred in infants who were hypersensitive to cows' milk. It was suggested that these infants had developed antibodies to cows' milk proteins absorbed from the alimentary tract [Parish *et al.*, 1960; Coombs *et al.*, 1982]. If a sleeping hypersensitive infant regurgitated some stomach contents which were aspirated in to the respiratory tract, death might result from an anaphylactic type of reaction. Evidence for this hypothesis came from experiments involving guinea pigs sensitised to cows' milk. The hypothesis was later disproved by a study which measured total and specific anti-cows' milk IgE levels in infants who died from SIDS and from infants who had had reported episodes of milk aspiration. If a SIDS infant had died from anaphylaxis, there should be increased levels of IgE. The study found that increased total and specific levels of IgE were only present in infants with a history of milk aspiration [Boulloche *et al.*, 1986]. Breast fed infants have also been found to die as suddenly and unexpectedly as those fed cows' milk [Berry *et al.*, 1989].

1.5.2 Metabolic disorders

A number of inborn errors of metabolism or metabolic deficiencies have been associated with SIDS [Bonham *et al.*, 1992; Burchell *et al.*, 1992]. Some metabolic disorders can mimic SIDS by their sudden onset and rapid deterioration [Bonham *et al.*, 1992]. One example is infants who have problems maintaining glucose homeostasis. These infants are subject to potentially fatal hypoglycaemic events and

this problem is enhanced during times of stress such as minor infections [Burchell *et al.*, 1992].

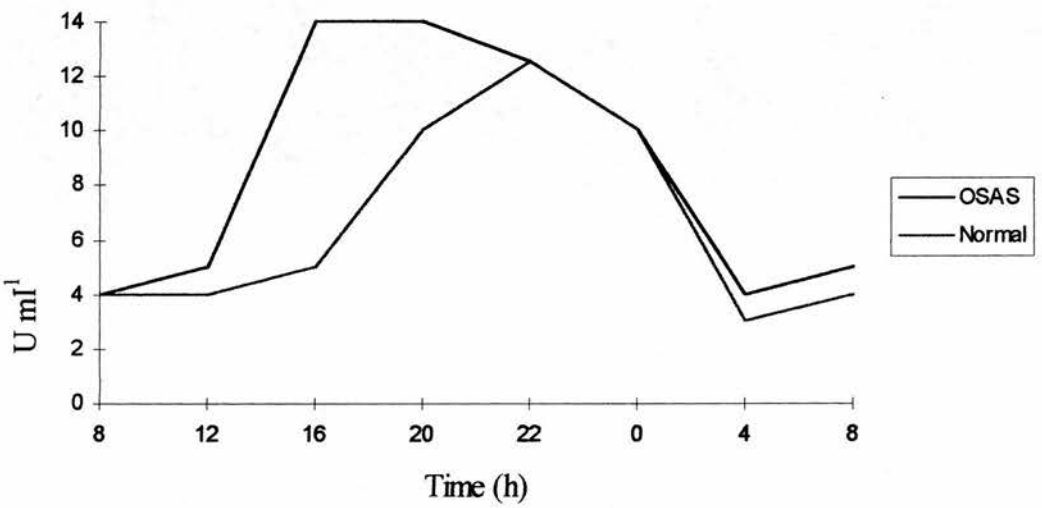
1.5.3 Sleep apnoea

Sleep apnoea has been proposed as an alternative mechanism of a SIDS death. Infants with sleep apnoea have abnormal respiratory control, airway obstruction and failure of arousal which results in death. Near-miss SIDS or infants who have had an apparently life threatening event (ALTE) have been followed to try to provide evidence for this hypothesis [Berry *et al.*, 1989].

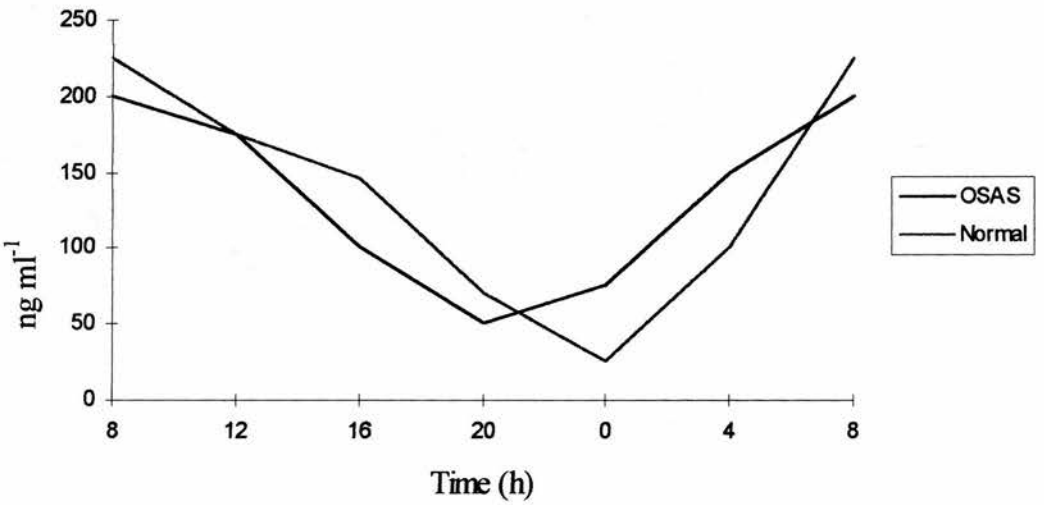
Studies of adults with obstructive sleep apnea syndrome (OSAS) found that these patients, compared to normal, healthy controls, had altered cytokine release patterns associated with circadian rhythm. Maximum release of cytokines from leukocytes of normal subjects in response to endotoxin occurred in the later evening or early hours of the morning and corresponded to minimal levels of cortisol. Leukocytes from OSAS subjects showed maximal release of cytokines during the afternoons which also corresponded to minimal cortisol levels (Figures 1.1a and b) [Entzian *et al.*, 1996].

Figure 1.1 a) interferon-gamma (IFN-γ) and b) cortisol release in OSAS patients and normal controls [Entzian *et al.*, 1996]

a)



b)



1.5.4 Virus infection and overheating

Infection and overheating are strongly associated with a SIDS death. A number of epidemiological factors provide evidence that virus infection is associated with SIDS; however, no definite association between SIDS and specific viruses have been reported, and it appears unlikely that virus infection alone is sufficient to cause death. Virus infection might contribute to overheating by induction of fever as a result of pyrogenic cytokine release in response to a primary viral or secondary bacterial infection [Fleming, 1992; Howatson, 1992].

Infants who had a virus infection and were heavily wrapped were found to be at an increased risk of sudden infant death [Gilbert *et al.*, 1992]. Physiological evidence indicates that infants in the 2 - 3 month age range are more vulnerable to heat stress than younger infants [Fleming *et al.*, 1992].

1.5.5 The common bacterial toxin hypothesis

The common bacterial toxin hypothesis is based on a mathematical model which was proposed to explain the mechanism of a SIDS death [Morris *et al.*, 1987]. The model is consistent with epidemiological and experimental studies that viral infections predispose to SIDS, there are minimal morphological changes at autopsy, maximum incidence during sleep, and the hypothesis closely predicts the observed SIDS age distribution.

The model states that early in life, infants encounter bacteria which form the normal flora of the respiratory tract. Infants are protected during this time by maternal IgG until they develop their own immunity to these bacteria and their toxins. The normal nasopharyngeal bacterial flora can, however, be disturbed by virus infection and this results in infants carrying increased numbers of toxigenic bacteria. The hypothesis states that if an infant with a viral infection is exposed to a toxigenic organism for the first time, the organism might overgrow in the nasopharynx and produce toxins which are absorbed across the respiratory mucosa. These toxins will stimulate strong inflammatory responses, and in a vulnerable infant, a SIDS death might result before immunity is developed. The hypothesis is based on the fact that the toxins are produced by bacteria of the normal flora and infants commonly encounter them during the first few weeks of life.

Nasopharyngeal bacterial flora and not gastrointestinal flora have been considered in this model because although the gastrointestinal tract contains many toxigenic bacteria, a strong association is observed between viral respiratory tract infections and SIDS.

The hypothesis closely predicts the observed age incidence of SIDS. Around 3 months of age, a baby's maternal immunoglobulins have declined and its active immunity will not be fully developed. The hypothesis also explains why premature compared to full term infants have an increased risk of dying from SIDS since premature infants have lower levels of maternal IgG in the first months of life.

The higher incidence of SIDS observed between midnight and 6.00 a.m. can also be explained. Gravity and mucociliary action normally clear nasopharyngeal secretions; however, if viral infection impairs mucociliary clearance, secretions can pool resulting in increased bacterial growth and toxin production during the later hours of sleep.

1.6 Infectious agents and SIDS

1.6.1 Infections prior to death

Prior to death, many SIDS infants had mild symptoms of respiratory or gastrointestinal infections [Lindsay *et al.*, 1994]. Infants are particularly susceptible to infections during the 2 - 4 month age range because levels of passive antibodies against bacteria and viruses are decreasing and infants are more reliant on their inflammatory responses to deal with infectious agents to which they are exposed.

1.6.2 Evidence of inflammatory responses in SIDS infants

There is evidence for mild infection and associated inflammatory responses in SIDS infants. As a group, SIDS victims had evidence of increased inflammatory stimulation in the upper airways and intestinal tract compared with infants who died of other causes (*e.g.* violent deaths). Pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) have been detected in body fluids or tissues of SIDS victims. At autopsy, there is

often evidence of mild inflammatory reactions and increased levels of some acute phase proteins, increased production of immunoglobulins in the respiratory tract and gut, and changes indicating T-cell activation [Forsyth *et al.*, 1999; Vege *et al.*, 1999].

1.6.3 Toxigenic bacteria isolated from SIDS infants

With the exception of an association between whooping cough and SIDS in some Scandinavian countries, [Lindgren *et al.*, 1997], there is no association between outbreak of disease and SIDS therefore it seems unlikely that one infectious agent is solely responsible for SIDS [Blackwell *et al.*, 1995a]. Bacteria which colonise mucosal surfaces and produce soluble toxins that diffuse into the bloodstream have formed the majority of the work involving the role of bacteria in SIDS [Blackwell *et al.*, 1995b].

There is evidence which indicates that the nasopharyngeal bacterial flora is disturbed in some SIDS infants [McKendrick *et al.*, 1992]. Compared to healthy infants, infants who died from SIDS have a significantly higher probability of having a toxigenic bacterial species isolated from their nasopharyngeal flora.

Toxigenic bacteria isolated more often from SIDS cases compared to controls include *Staphylococcus aureus*, *Streptococcus pyogenes*, *Clostridium perfringens*, *Clostridium difficile* and toxigenic *Escherichia coli* [Telford *et al.*, 1989; Bettelheim *et al.*, 1990; Murrell *et al.*, 1993; Bettiol *et al.*, 1994; Lindsay *et al.*,

1994]. It has also been suggested that enteric staphylococcal infection is involved in some SIDS deaths [Trube-Becker, 1978]. *Bordetella pertussis* has been implicated by epidemiological studies as a possible cause of SIDS [Wennegren *et al.*, 1987; Nicoll *et al.*, 1988; Lindgren *et al.*, 1997]. The numbers of SIDS cases in Scandinavia increased following discontinuation of the pertussis vaccine [Wennegren *et al.*, 1987]. *B. pertussis* has not been isolated from any cases of SIDS [Saadi *et al.*, 1996a; Heinenger *et al.*, 1996], but DNA from these bacteria was identified in 9/51 (18%) of SIDS infants. The study was not carried out in parallel with attempts to culture the bacteria [Heininger *et al.*, 1996].

In a recent study, *S. aureus* was the species most frequently isolated from SIDS infants. *S. aureus* was isolated from 56% of 253 healthy infants aged 3 months or younger compared to 86.4% of SIDS infants in the same age range. A large number of SIDS deaths now occur during the first 3 months of life, and three studies have found that in the normal flora of the respiratory tract of infants, *S. aureus* was the predominant isolate during the 0 - 3 month age range [Aniansson *et al.*, 1992; Blackwell *et al.*, 1999; Harrison *et al.*, 1999a]. In infants with and without an upper respiratory tract infection, *S. aureus* was found to be more common during the autumn and winter months compared to the spring and summer months [Harrison *et al.*, 1999a]. This parallels the winter peak of SIDS noted in many countries [Blackwell *et al.*, 1995a]. Although male infants compared to female infants were not more likely to be colonised by the respiratory pathogen *S. aureus* which has been implicated in SIDS, male infants had significantly heavier growth [Harrison *et*

al., 1999a]. In this study, compared to female infants, male infants who slept prone were found to have significantly higher counts of *S. aureus* regardless of the presence or absence of an upper respiratory tract infection.

1.6.4 Bacterial toxins associated with SIDS

Several groups have proposed that bacterial toxins play a role in precipitating the series of events leading to SIDS [Morris *et al.*, 1987; Blackwell *et al.*, 1992; Lindsay *et al.*, 1994; Blackwell *et al.*, 1995a; Blackwell *et al.*, 1995b]. Three bacteria commonly isolated from SIDS infants, *S. aureus*, *S. pyogenes* and *C. perfringens*, produce superantigenic toxins which bind to MHC class II molecules and activate Vβ-specific T cells [Johnson *et al.*, 1996]. These toxins have been implicated as having a significant role in SIDS [Lindsay *et al.*, 1994].

The *S. aureus* toxins, toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin C (SEC), have been identified in tissues of SIDS infants by immunohistochemical methods [Newbould *et al.*, 1989; Malam *et al.*, 1992]. By ELISA and flow cytometry methods, pyrogenic staphylococcal toxins have been identified in over half of the specimens from SIDS infants from Scotland, France and Australia examined in a recent study [Zorgani *et al.*, 1999].

C. perfringens and its enterotoxin A have been identified in body fluids of SIDS infants [Murrell *et al.*, 1987], and a significantly higher incidence of both were

found in the faeces of formula fed infants compared to breast fed infants [Murrell *et al.*, 1993].

The common bacterial toxin hypothesis suggested that SIDS might be caused by a number of different toxins which could act synergistically [Morris *et al.*, 1987, 1999]. The chick embryo model has been used to examine the lethality of toxins produced by bacteria associated with SIDS. Synergy between Gram-positive and Gram-negative bacteria (*S. aureus* and *E. coli*) has been demonstrated [Drucker *et al.*, 1992]. A later study [Sayers *et al.*, 1995a] showed that synergy can occur between the toxins of nasopharyngeal staphylococci and enterobacteria and that this combination of strains is more likely to occur in the nasopharynx of SIDS victims than in healthy infants. The pyrogenic staphylococcal toxins have been demonstrated to enhance the effect of endotoxin over 100,000 fold [Bohach *et al.*, 1990]. While high levels of endotoxin have not been identified in SIDS infants [Platt *et al.*, 1994; Crawley *et al.*, 1999], in combination with pyrogenic toxins of staphylococci or streptococci, these might have lethal effects.

Other animal models indicate virus infection can enhance inflammatory responses to staphylococcal toxins [Jakeman *et al.*, 1991; Sarawar *et al.*, 1994; LeClaire *et al.*, 1995]. This enhancement effect is due to priming of inflammatory responses (TNF and nitric oxide) by IFN-γ produced in response to the viral infection [Sarawar *et al.*, 1994; LeClaire *et al.*, 1995]. Influenza virus infection of human leukocytes resulted

in increased production of TNF-α in response to endotoxin [Lundemose *et al.*, 1993].

1.7 Factors that contribute to susceptibility to SIDS

1.7.1 Exposure to cigarette smoke

Maternal smoking whether ante- or post-natal, is a risk factor for SIDS. In a recent epidemiological study, the risk of SIDS increased with the number of parents who smoked and with the number of cigarettes smoked. The risk of SIDS caused by maternal smoking was increased if the mother shared a bed with the infant [Mitchell *et al.*, 1995; Brooke *et al.*, 1997].

Smoking is associated with respiratory viral infections which enhance bacterial binding, and buccal epithelial cells from smokers have been found to bind significantly more bacteria compared to cells from non-smokers [Saadi *et al.*, 1996a; El Ahmer *et al.*, 1999]. The studies by El Ahmer *et al*; [1999] indicate a broad range of bacteria exhibited this pattern including species implicated in SIDS (*S. aureus* and *B. pertussis*) and species that cause respiratory tract infections (*Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*).

Buccal epithelial cells from non-smokers treated with a water soluble cigarette smoke extract (CSE) demonstrated enhanced bacterial binding. The effect was observed up to a 1 in 300 dilution of the extract. Passive exposure to cigarette

smoke might result in adsorption of substances that enhance attachment of bacterial species. Passive coating of an infant's mucosa by components of cigarette smoke might increase the non-specific "stickiness" of these cells for bacteria.

A significant association has been found between isolation of specific bacterial species from both mother and infant [Blackwell *et al.*, 1999]. The mother is probably the source of an infant's normal flora and the significantly enhanced binding of bacteria to buccal cells of adult smokers might result in the infant being exposed to a greater number of bacteria.

Sayers *et al.* [1995b] showed that very low concentrations of nicotine can interact with selected bacterial toxins and potentiate the lethal effect of both single toxins and synergistic combinations. This might also explain why parental smoking increases the risk of SIDS. Cigarette smoke components were found to induce inflammatory responses on their own and could enhance inflammatory responses to virus infection [Raza *et al.*, 1999].

Low birthweight is also associated with an increased risk of SIDS and a recent study has suggested that most of the SIDS risk associated with growth retardation might be due to maternal smoking [Cooke, 1998].

1.7.2 The prone sleeping position

Infants sleeping prone were found to be at 3 times the risk of SIDS compared to infants in other sleeping positions [Beal, 1995]. The incidence of SIDS has declined in response to campaigns to discourage prone sleeping [Beal, 1995; Court, 1995; Molony *et al.*, 1998].

Pyrogenic toxins of *S. aureus* have been identified in the tissues and body fluids of SIDS infants [Newbould *et al.*, 1989; Malam *et al.*, 1992; Zorgani *et al.*, 1999]. These toxins are induced only between 37 and 40°C, and the nasal temperature is usually well below this range due to the passage of air. In the prone position, there could be an accumulation of secretions or blocking of a nostril by bedding which would create a microenvironment allowing increased bacterial growth and toxin production. Infants sleeping prone also have a reduced ability to lose heat compared to infants sleeping supine [Tuffnell *et al.*, 1995].

The temperatures in the noses of young children were found to be significantly higher than those of adults [Molony *et al.*, 1996]. In a recent study, the nasal temperatures of children lying in the prone position were significantly higher than in the standing position. The temperature in the prone position, but not in the upright position, reached 37°C in 5 children who had no evidence of a respiratory tract infection [Molony *et al.*, 1998; Molony *et al.*, 1999]. If the prone position can raise the upper airway surface temperature to the range in which pyrogenic toxins are

produced, this might be one mechanism by which prone sleeping contributes to SIDS in the absence of a respiratory tract infection [Molony *et al.*, 1998].

The hypothesis that the prone sleeping position is associated with accumulation of upper airways secretions and increased bacterial growth was investigated in adults. In a study in which 10 adults with upper respiratory tract infections lay prone for one hour and then supine for one hour, higher bacterial counts were obtained from nasal swabs after the prone position. In the prone sleeping position, the clearance of upper respiratory tract secretions is reduced leading to increased bacterial growth and potentially to increased toxin production [Bell *et al.*, 1996].

A recent study found that infants who had an upper respiratory tract infection and slept prone had significantly higher numbers of bacteria in their secretions and a similar nasopharyngeal flora to that observed in SIDS infants [Harrison *et al.*, 1999a]. These observations are consistent with the common bacterial toxin hypothesis [Morris *et al.*, 1987, 1999] which suggests that if an infant with an upper respiratory tract infection is placed in the prone sleeping position, increased bacterial growth and toxin production might result in an infant being at an increased risk of a SIDS death [Harrison *et al.*, 1999a, 1999b].

1.7.3 Sleep and developmental changes in relation to SIDS

Most SIDS deaths occur between midnight and 6.00 am [Morris *et al.*, 1987] when babies are asleep, and it has been suggested that sleep might be a part of the

pathophysiological mechanism of SIDS [Schechtman *et al.*, 1995; Cornwell *et al.*, 1998]. Studies on the physiology of sleep in infants have produced important clues to relate developmental stage with the proposed role of inflammation in SIDS.

The 2 - 4 month age range has been consistently identified as the period in which the majority of SIDS deaths occur. During this age range, infants develop adult-like night-time temperature and hormone rhythm patterns. In normal infants, between 7 and 16 weeks, the body temperature falls at night to 36.4°C, similar to that of sleeping adults. Breast fed infants undergo this change significantly earlier than formula fed infants. Among infants of low birthweight or who experienced problems at delivery, this change can occur as late as 22 weeks [Lodemore *et al.*, 1992]. Infants who remain in the "immature" state share many risk factors with SIDS infants, and Wailoo and colleagues have suggested that the "immature" state is a risk factor for SIDS. In contrast, other work by this group found that Asian infants stay in the "immature" stage significantly longer than white infants [Petersen *et al.*, 1994], but Asian infants have a lower incidence of SIDS [Balarajan *et al.*, 1989].

The findings of Wailoo and colleagues can also be explained by relating them to other physiological changes they found to occur in conjunction with the change in body temperature rhythm. The week following the switch to an adult-like night-time temperature pattern, there is a dramatic drop in night-time (but not day-time) levels of the anti-inflammatory agent cortisol. In adults, inflammatory responses are less

well controlled at night due to the circadian rhythm pattern of cortisol. Lowest levels of cortisol occur during the early hours of the morning [Entzian *et al.*, 1996].

The period in which the drop in night-time cortisol levels occurs is thought to be a window of vulnerability for SIDS. If the change occurs when maternal antibodies are present to neutralise viruses or toxins (or the infant has produced its own antibodies), the infant can deal with infectious challenges. If this drop in night-time cortisol levels occurs in an infant who has little or no specific protection, the infant is at increased risk of dying from SIDS due to an uncontrolled immune response [Blackwell *et al.*, 1995a, b]. Evidence that younger infants control inflammatory responses more effectively, comes from studies carried out in relation to trials for the change in immunisation schedules in Britain. This evidence was that younger infants had fewer episodes of high temperature and fewer local inflammatory reactions [Miller *et al.*, 1997].

1.8 Factors that reduce susceptibility to SIDS

1.8.1 Immunisation with diphtheria-pertussis-tetanus vaccine

Infants who were not immunised or who were immunised late against diphtheria, pertussis and tetanus (DPT) were found to be at a greater risk of SIDS. The NICHD SIDS Co-operative Epidemiological Study found that there was a significantly lower incidence of immunisation among SIDS infants compared to control infants [Hoffman *et al.*, 1987].

In 1990, the DPT immunisation schedule in Britain was changed; immunisation was initiated at 2 months rather than 3 months. The DPT vaccine can induce antibodies which cross-react with staphylococcal pyrogenic enterotoxins [Essery *et al.*, 1999]. The new immunisation schedule might prevent a few deaths due to asymptomatic whooping cough but they might also induce production of antibodies which are cross-reactive with adhesins or toxins of other species during a period in which infants appear to be better able to handle their inflammatory responses.

1.8.2 Breast feeding and SIDS

The first association between SIDS and the complete absence of breast feeding was noted by Carpenter in 1965 [cited in Klonoff-Cohen *et al.*, 1995]. The actual protective mechanism of breast feeding in relation to SIDS is not known; however, breast fed infants have a lower risk of SIDS and breast feeding promotes an infant's general health and well-being.

Breast milk is specifically made for humans and because of its anti-infective, nutritional and biological properties, has been characterised as the optimal food for human growth and development [Uauy *et al.*, 1995; Golding *et al.*, 1997]. Breast milk is a dynamic body fluid and its composition varies both during the day and over the course of lactation thus providing the infant with the specific nutrients it needs at each age [Lawrence, 1994; Emmett *et al.*, 1997]. It is not presently understood what the physiological basis of this variation is in relation to infant development

[Emmett *et al.*, 1997]. Breast feeding has also been found to protect against the development of diseases such as insulin dependent diabetes mellitus later in life [Lambert *et al.*, 1995].

Breast milk is used as the standard by which infant formulas are improved [Jensen *et al.*, 1992]; however, although nutritionally complete, infant formula does not contain protective immunological factors, and it is not possible to provide either the type and quality of the individual nutrients or the day to day variations observed in breast milk.

1.8.2.1 Evidence for a protective effect of breast feeding in relation to SIDS

Epidemiological studies, including the New Zealand Cot Death Study which was carried out over a period of 3 years and examined data from 465 SIDS cases and 1,762 controls [Mitchell *et al.*, 1991; Mitchell *et al.*, 1992], indicated that exclusive breast feeding, compared with partial breast / formula or exclusive formula feeding, provided a protective effect against the risk of SIDS. Infants not exclusively breast fed on discharge from hospital were twice as likely to die from SIDS compared to infants who were exclusively breast fed [Ford *et al.*, 1993]. This figure was also found in a case-control study conducted by the National Institute of Child Health and Human Development SIDS Co-operative Epidemiological Study in the US [Hoffman *et al.*, 1988]. This reduced risk persisted during the first 6 months of life after controlling for confounding demographic, maternal and infant factors [Ford *et al.*, 1993].

The reduced risk of SIDS in breast fed infants was evident during the first 6 months of life. Higher levels were found for control infants compared to SIDS infants, and compared to control infants, breast feeding stopped earlier in SIDS cases. During the first week of life, 92% of control infants were breast fed compared with 86% of SIDS cases. By 6 weeks, the numbers were 77% and 63% respectively and by 6 months, the numbers were 58% and 36% respectively. This study could not identify a causal relationship between the reduced risk of SIDS and breast feeding, but it has been suggested that the protective effect might be due to the breast milk itself, to the environment which has led to a continuation of breast feeding, or to a combination of both [Ford *et al.*, 1993].

Contradictory reports came from a UK study which investigated the hypothesis that bottle feeding is associated with SIDS. Compared with fully breast fed babies, the crude odds ratio for SIDS among fully bottle fed babies was 3.1 and for mixed breast and bottle fed infants 1.5. Ratios fell to 1.8 and 1.2 respectively after adjustment for maternal smoking, parental employment, pre-term gestation and sleeping position. The conclusion of the British study was that bottle feeding was not a significant independent risk factor for SIDS and that most of the apparent associations between bottle feeding and SIDS could be accounted for by maternal smoking, pre-term gestation and parental employment status [Gilbert *et al.*, 1995]. These results have, however, been criticised on the basis of design and small numbers. Only larger studies tend to demonstrate an independent risk of bottle

feeding associated with SIDS. A second major design flaw of the study was biased controls. To recruit control infants, health visitors were asked to identify two infants living in the same neighbourhood; however, these controls would be more likely to have the same feeding method compared to randomly selected controls. This could account for the fact that bottle feeding was not found to be independently associated with SIDS. One other factor which could account for this finding is that when the limited format of an observational case-control study is used, adjustment for socioeconomic status might remove the effect of breast feeding [Tappin, 1995]. In Britain, breast feeding tends to be confined to the upper and middle classes, whereas in countries such as New Zealand, women of all socioeconomic groups breast feed [Tappin, 1995]. In a longitudinal study of mothers and infants, smoking and formula feeding were found to be significantly higher among lower socioeconomic groups [Blackwell *et al.*, 1999].

1.8.2.2 General protective effects of breast feeding

In comparison to bottle fed infants, breast fed infants are less susceptible to gastrointestinal and respiratory tract infections [Buescher, 1994; Pisacane *et al.*, 1994]. Breast feeding has been found to be protective against specific pathogens including *Salmonella*, *Shigella*, *Vibrio cholerae*, *Escherichia coli*, polioviruses, rotavirus and respiratory syncytial virus (RSV) [Goldman, 1993]. This protection might also be applicable to *S. aureus* and *C. perfringens*, toxigenic bacteria which colonise the respiratory and gastrointestinal tracts respectively and have been implicated in SIDS [Lindsay *et al.*, 1994; Blackwell *et al.*, 1995a]. Secretory IgA

from human colostrum has also been found to inhibit adherence of *H. pylori* to the human gastric mucosa [Lönnerdal, 1996].

1.8.2.2.1 Immune factors in human milk

It has been suggested that breast milk confers general and specific protection upon an infant. Antibody-producing cells specific for the mother's microbial flora are secreted in breast milk. The infant acquires its mucosal flora from the mother. There was a significant association between isolation of potential pathogens such as *S. aureus* or *H. influenzae* from both mother and baby [Mackenzie *et al.*, 1996; Blackwell *et al.*, 1999]. The infant's respiratory defences might be stimulated by immunoregulatory factors present in human milk [Cunningham *et al.*, 1991]. Secretory IgA and cells of the immune system present in breast milk could enhance this protective effect.

Breast milk contains a number of antimicrobial factors including lactoferrin, lysozyme and secretory IgA. These factors are common to mucosal sites, are able to survive in the gastrointestinal tract because of their resistance to digestive enzymes and can protect without triggering inflammatory reactions [Goldman, 1993].

Breast milk also contains a number of anti-inflammatory agents including enzymes that degrade mediators of inflammation. In studies with a rat subcutaneous air pouch model of inflammation, colostrum was found to have significant anti-inflammatory activity [Murphey *et al.*, 1993]. In a later study involving chemically induced colitis in rats, human milk was found to have an anti-inflammatory effect compared to rats

fed infant formula. This might have been due to the presence of the IL-1 receptor antagonist in human milk [Grazioso *et al.*, 1997]. Human milk also contains the anti-inflammatory cytokine IL-10. This has been found to regulate mucosal defences and limit inflammatory reactions in the upper part of the alimentary and respiratory tracts [Garofalo *et al.*, 1995].

Breast milk also contains growth factors including epithelial growth factor, lactoferrin, cortisol and immunomodulating agents [Goldman, 1993]. The cytokines IL-1β and TNF-α have been identified in colostrum and human milk. These have been suggested to activate T cells and enhance secretory component production respectively [Rudloff *et al.*, 1991; Saito *et al.*, 1991; Goldman, 1993]. Both IL-6 (which enhances IgA production in the breasts) and granulocyte-colony stimulating factor are involved in the activation and maturation of the immune system, and these might help to protect the breast fed infant against pathogenic organisms. The presence of these cytokines could contribute to the lower infection rate in breast fed infants [Wallace *et al.*, 1997].

1.8.2.2.2 IgA

Secretory IgA is the main immunoglobulin in breast milk and is made up of an IgA dimer linked to glycoprotein produced by epithelial cells. Many of the IgA antibodies are specific for microorganisms in the respiratory system or gastrointestinal tract. This specificity is due to immunogen-triggered migration of B cells from Peyer's patches of the lower small intestine and from lymphoid centres in

the bronchial tree to the lamina propria of the mammary gland [Goldman, 1993]. Compared to bottle fed infants, breast fed infants have enhanced immunity on their mucosal surfaces during the early neonatal period as development of the secretory IgA system does not occur until sometime after birth; however, it has been shown that by 6 weeks, local antigens are the main source of stimulation for immunoglobulin production in the respiratory mucosa [Stephens, 1986; Goldman, 1993].

1.8.2.2.3 Oligosaccharides in human milk

The initial event in development of most bacterial diseases is attachment to mucosal surfaces [Beachey, 1981]. Human milk contains a unique set of complex oligosaccharides, and because of similarities with epithelial cell-surface carbohydrates, both free oligosaccharides and glycoconjugates in human milk have been suggested to act as receptor analogues for some bacterial adhesins [Kunz *et al.*, 1993]. These might increase the resistance of breast fed infants to infection by binding to bacterial adhesins thereby reducing the ability of respiratory and gastrointestinal pathogens to colonise epithelial surfaces [Sabharwal *et al.*, 1991; Goldman, 1993; Kunz *et al.*, 1993; Ashkenazi, 1996]. Human milk oligosaccharides have been found to inhibit binding of *S. pneumoniae* and *H. influenzae* to epithelial cells [Andersson *et al.*, 1986] and to inhibit binding and block activity of the heat stable enterotoxin of *E. coli* [Crane *et al.*, 1994].

The concentration of oligosaccharides in human milk are highest early in lactation [McVeagh, 1995]. Oligosaccharides are present in colostrum at 2.1 g/100 ml and in mature milk at 1.3 g/100 ml [Emmett *et al.*, 1997]. Breast milk contains over 130 different forms and the variety of oligosaccharides differs from woman to woman [McVeagh, 1995].

The Lewis antigens can occur as free oligosaccharides in breast milk [Henry *et al.*, 1995]. Oligosaccharides in milk are related to the mother's secretor status [McVeagh, 1995]. Milk from women who secrete their ABO blood group antigens are characterised by the presence of 2-fucosyl-lactose (Fucα1-2Galβ1-4Glc) and of lacto-N-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc) and of more complex oligosaccharides, all possessing Fucα1-2Galβ1-3GlcNAc residues. In milk of non-secretors, the major fucosylated oligosaccharide is lacto-N-fucopentaose II (Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc) which is the Lewis^a blood group component (Figure 1.2) [Kunz *et al.*, 1993].

The Lewis antigens are fucose containing oligosaccharides that can act as receptors for several bacteria: *H. pylori* [Alkout *et al.*, 1997]; *S. aureus* [Saadi *et al.*, 1993, 1999]; *B. pertussis* [Saadi *et al.*, 1996a]. The Lewis antigens are not structural components of cells but are rather acquired by cells from plasma or secretions. Expression of the Lewis antigens depends on the Lewis (on the short arm of chromosome 19) and secretor loci. The Lewis gene encodes for an α-1,3/4-fucosyl-transferase which transfers fucose to the subterminal βGlcNAc unit of precursor

chains. The secretor gene codes for an α-1,2-fucosyl-transferase that transfers fucose to the terminal βGal unit of precursor chains [Henry *et al.*, 1995].

Fucosyltransferases coded by genes at these loci compete and interact with each other and with other transferases to determine an individual's Lewis and secretor phenotype [Henry *et al.*, 1995]. If the secretor enzyme adds fucose first, the structure can act as a substrate for the Lewis enzyme to form the Lewis^b antigen; however, if the Lewis enzyme adds fucose to the subterminal sugar first to form Lewis^a, the secretor gene is unable to use this as a substrate to form Lewis^b. Figure 1.3 outlines the synthesis pathways of the Lewis^a and Lewis^b antigens.

Figure 1.2 The structure of the Lewis antigens [Henry *et al.*, 1995]

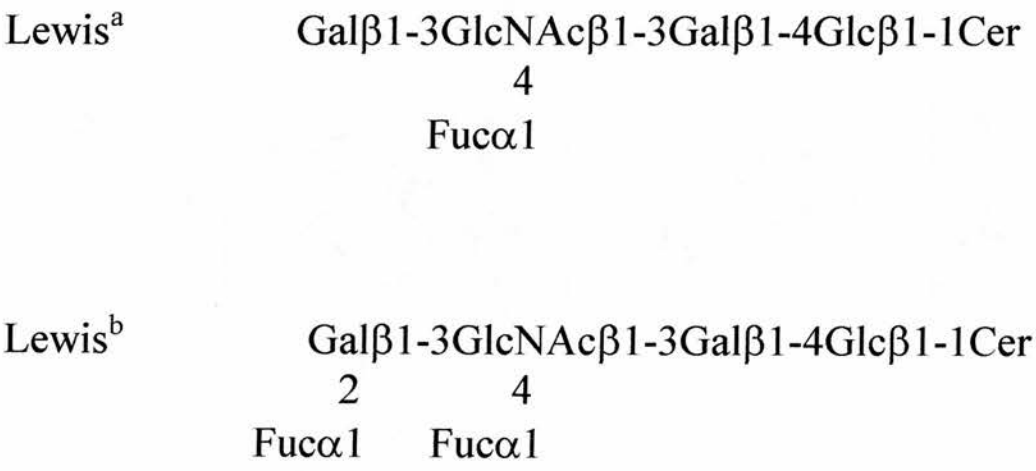
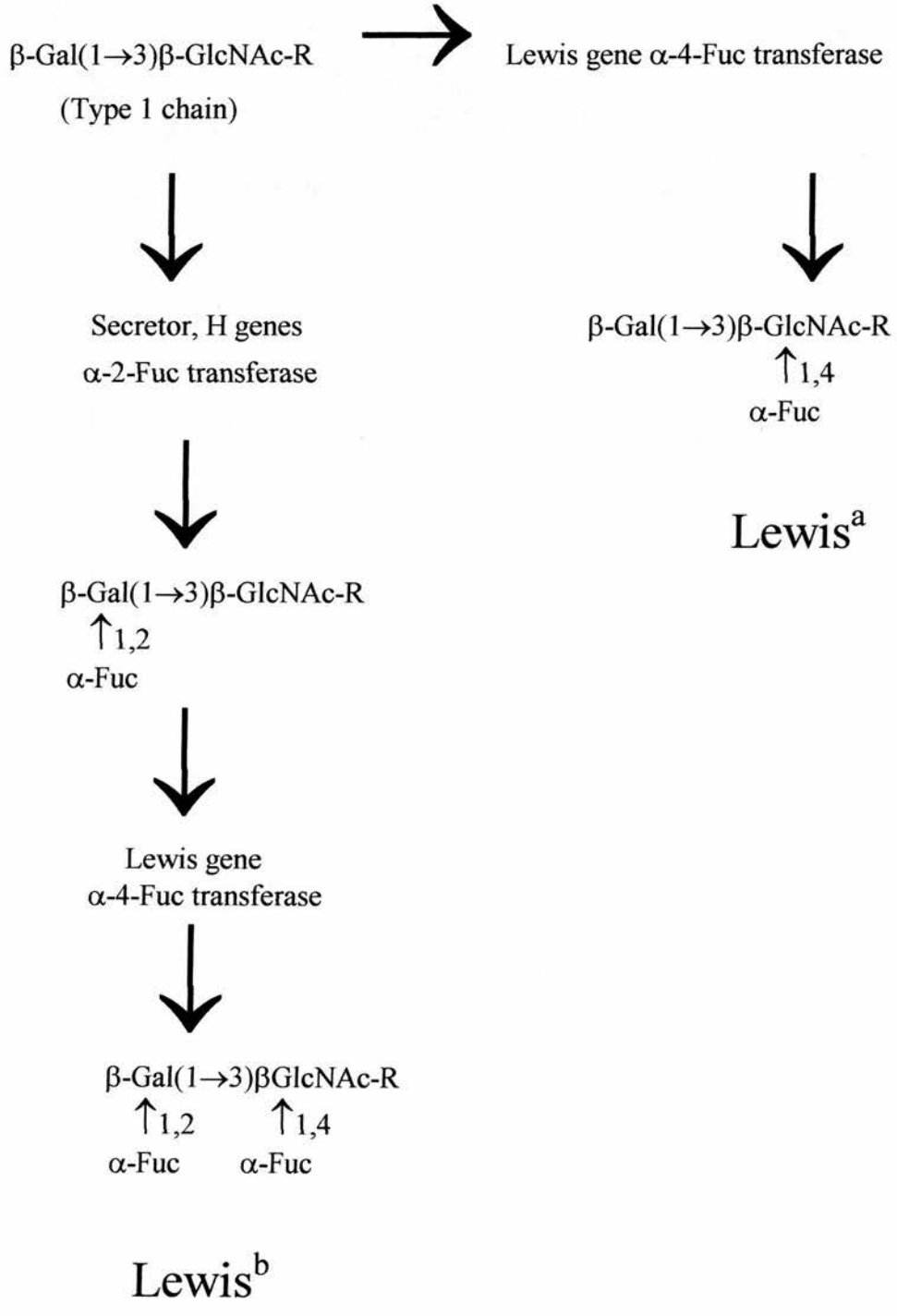


Figure 1.3 The synthesis pathways of the Lewis[a] and Lewis[b] antigens [Watkins, 1980]



1.8.2.2.4 Effect of breast feeding on neurological development

Infants fed different nutrient mixtures have been found to have important biochemical and physiological differences. Low birth weight infants were found to have significant differences in fundamental autonomic functions and this could be related to dietary intake. Studies have also found that depending on the quality of lipid in the diet, significant differences were found in the visual responses of neonates fed human milk versus formula [Schulze, *et al.*, 1995].

Breast milk has been suggested to contribute to optimal brain development [Uauy *et al.*, 1995]. Long-chain polyunsaturated fatty acids such as arachidonic and docosahexaenoic acid are essential for brain development [Lanting *et al.*, 1994]. Brain growth is associated with increased incorporation of these fatty acids into the phospholipid which is primarily located in the cerebral cortex [Farquharson *et al.*, 1992]. During periods of rapid neuronal multiplication, the placenta supplies the foetus with arachidonic and docosahexaenoic acids [Kohn *et al.*, 1994; Lanting *et al.*, 1994]; however, after birth, young infants are unable to synthesis these fatty acids from their C18 precursors quickly enough and, therefore, require an external source [Emmett *et al.*, 1997]. These fatty acids are present in breast milk but not in infant formula. Compared to breast fed infants, the plasma docosahexaenoic acid levels are much lower in formula fed infants [Uauy *et al.*, 1995]. It has been suggested that infant formula could be improved by the addition of long chain fatty acids [Farquharson *et al.*, 1992]

One study investigated the phospholipid fatty acid composition of the cerebral cortex from SIDS infants and split them into groups depending upon whether they were fed breast milk or infant formula. Although small, this study found that breast fed infants had significantly higher concentrations of docosahexaenoic acid in their cerebral cortex than formula fed infants [Farquharson *et al.*, 1992].

1.8.2.2.5 Disadvantages of breast feeding

Breast feeding has some disadvantages. Breast milk might contain any maternal medication or drugs ingested (e.g. caffeine, nicotine, alcohol), and viruses including Epstein-Barr virus (EBV), cytomegalovirus (CMV) and HIV, can be passed from mother to infant [Golding *et al.*, 1997].

It has been found that the protective effect of breast feeding against SIDS is negated among infants exposed to maternal tobacco smoke [Klonoff-Cohen *et al.*, 1995]. Compared to passive smokers, breast milk from mothers who smoke has a much higher concentration of cotinine [Milnerowicz *et al.*, 1997]. An earlier study found that this depended on the mother's nicotine consumption and that babies exclusively breast fed by smoking mothers had urinary cotinine excretion levels in the same range as those of adult smokers [Schulte-Hobein *et al.*, 1992].

Infant botulism was suggested to be a cause of some SIDS deaths. Although the toxin responsible for botulism can be passed on to an infant in breast milk [Golding *et al.*, 1997], breast milk has been found to contain secretory IgA antibodies against

Clostridium botulinum vegetative cell antigens and breast fed infants compared to formula fed infants were found to have a slower onset of disease [Arnon, 1984].

1.9 The mechanism of a SIDS death

A number of hypotheses have been proposed to explain the mechanism of a SIDS death and are listed in Table 1.2. The most relevant to this project is that some deaths result from pathophysiological responses, produced against a combination of microbial products and / or cigarette smoke, occurring during a time of an infant's development when the endocrine responses are less able to "damp down" the effects of inflammatory mediators [Blackwell *et al.*, 1995a, b].

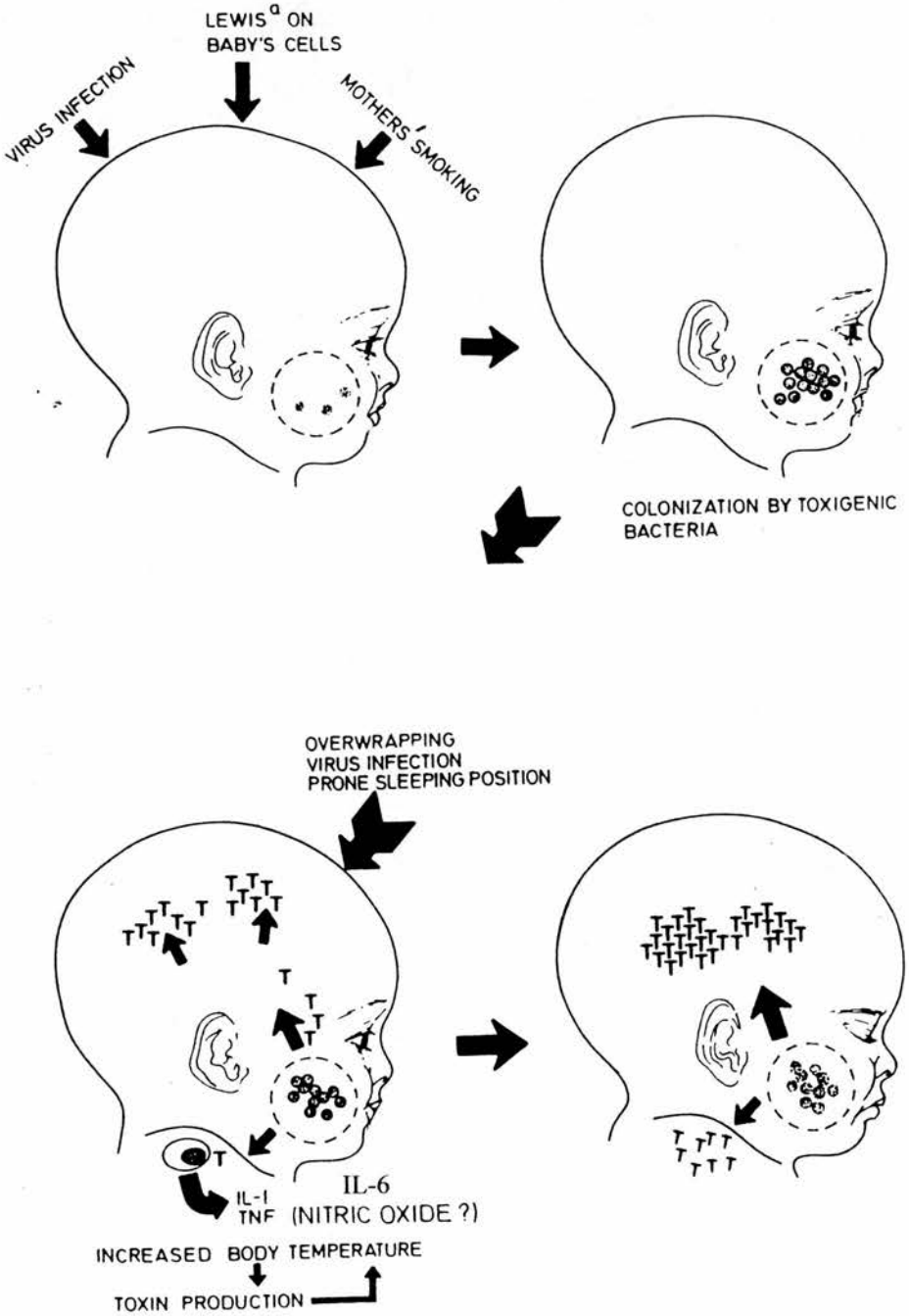
The model in Figure 1.4 outlines the steps leading to a SIDS death. The model was developed for respiratory pathogens but can also be applied to gastrointestinal pathogens. There are a number of factors which enhance colonisation of an infant by bacteria: virus infection; maternal smoking; expression of the Lewis^a antigen on baby's cells.

The Lewis^a antigen can be used as a host cell receptor by bacteria implicated in SIDS including the respiratory pathogens *S. aureus* and *B. pertussis* [Saadi *et al.*, 1993; 1994; 1996a, b]. During the 2 - 4 month age range, 80-90% of infants express Lewis^a antigen on their red blood cells, and the antigen is also present on epithelial cells since it can be adsorbed from secretions. There are differences in efficiency and maturation of the Lewis and secretor genes during this period of an infant's

development. The secretor gene matures later than the Lewis gene therefore at this time, even secretor infants will express high amounts of Lewis^a on their cells [Blackwell *et al.*, 1992]. The Lewis^a antigen was found in respiratory secretions of 71% of SIDS infants [Blackwell *et al.*, 1992; Saadi *et al.*, 1993].

If an infant is over-wrapped, subject to virus infection or placed in the prone sleeping position, the temperature at the mucosal surface might rise to between 37 and 40°C allowing production of superantigenic toxins. These toxins induce production of inflammatory mediators, and if the infant is unable to control inflammatory responses by 1) antibodies neutralising the toxin or 2) by sufficient cortisol responses, this could lead to uncontrolled inflammatory responses similar to those involved in toxic shock syndrome.

Figure 1.4 Model to outline the steps leading to a SIDS death



1.10 Aims of the project

The actual protective mechanism of breast feeding in relation to SIDS is not known. The aims of this project were to investigate a possible protective effect of breast feeding at two stages of the model: 1) at the level of colonisation by bacteria; 2) at the level of induction of inflammatory mediators to bacterial toxins.

The specific objectives were to address the following questions:

1) Do the methods used in most binding studies reflect the interactions at the mucosal surface?
2) What were the effects of human milk and infant formula on binding of a respiratory and a gastrointestinal pathogen implicated in SIDS to epithelial cells?
3) What were the effects of oligosaccharide components on bacterial binding to epithelial cells?
4) Are there antibodies to the toxins implicated in SIDS present in human milk?
5) Can components in human milk reduce the ability of the staphylococcal toxins to induce inflammatory mediators?
6) Are the night-time levels of cortisol sufficient to control inflammatory responses to staphylococcal toxins?

Although bacterial toxins have been detected in tissues from some SIDS infants and inflammatory responses observed, there is at present no direct evidence that the

toxins have induced the observed inflammatory responses. As part of this project, the last objective was to develop a reverse transcriptase polymerase chain reaction (RT-PCR) protocol to provide a method to obtain evidence that toxins in tissues of SIDS infants have induced the inflammatory responses.

Chapter Two

General materials and methods

2.1 Phosphate buffered saline (PBS)

This was prepared by addition of 6.8 g NaCl (Sigma), 1.43 g Na_2HPO_4 (BDH) and 0.43 g KH_2PO_4 (Fisons) to 1 litre of distilled water (pH 7.2).

2.2 Kato III cell culture and collection

Kato III cells were chosen for use in this model system to minimise differences in expression of H and Lewis antigens noted for individual donors as variation associated with diet or smoking [Alkout *et al.*, 1997]. Kato III, an epithelial cell line derived from a gastric carcinoma (CB769) was obtained from the European Collection of Animal Cell Cultures. Cells were cultured in 75 cm^3 tissue culture flasks (Greiner) at 37°C in 5% CO_2 in growth medium (GM) which contained RPMI 1640 (Gibco), 20% (v/v) foetal calf serum (FCS) (Gibco), 1% (w/v) L-glutamine (Gibco), penicillin (100 IU ml^{-1}) and streptomycin (200 μg ml^{-1}) (Gibco).

For binding assays, cells were detached from the flask by gentle scraping using a cell scraper (Greiner). The cells were washed twice in PBS by centrifugation at 300 x *g* for 10 min and resuspended in PBS. The cells were counted using a Neubauer haemocytometer by preparing a 1 in 10 dilution of the cells in 0.5% (w/v) trypan blue (Northumbria Biological, UK). The cell number was adjusted to 5 x 10^5 cells ml^{-1} with PBS.

2.3 Culture of bacteria

S. aureus NCTC 10655 which produces TSST-1 was grown on nutrient agar plates for 24 h at 37°C. *C. perfringens* NCTC 2661 (kindly provided by Mr R. Brown of this department) was grown on blood agar plates for 24 h at 37°C under anaerobic conditions (Oxoid Gas Generating Kit).

2.4 Collection of milk specimens and preparation of infant formula

Milk was collected from mothers participating in a longitudinal survey of infants' nasopharyngeal flora. Ethical permission for the study was obtained. Informed written consent was given by the mothers at recruitment into the study by the research nurse and health visitors at the 6 week postnatal medical examination. Information on socioeconomic background and medical history was obtained by the research nurse who helped the mother fill in a standardised questionnaire at the first interview.

Milk specimens were collected from women in various stages of lactation by manual expression. The specimens were transported within 2 - 4 h to the laboratory and stored at -20°C until examined. Whole and defatted pools of human milk containing aliquots of 42 specimens were prepared. To prepare the defatted pool, an aliquot of the whole pool was centrifuged twice at 2,000 x *g* for 15 min at 4°C. The clear middle layer was separated from the upper fatty layer and from cells at the bottom of the tube. Both pools were stored in aliquots at -20°C.

Infant formula was prepared with sterile water according to the manufacturers' instructions. All dilutions of milk and infant formula were made in PBS.

2.5 ELISA reagents

Table 2.1 lists the antibodies, horse radish peroxidase (HRP)-labelled antibodies, the standards and their sources.

2.5.1 Coating buffer

Coating buffer was prepared by addition of 1.59 g Na_2CO_3 (BDH), 2.93 g $NaHCO_3$ (BDH) and 0.20 g NaN_3 (Sigma) to 1 litre of distilled water (pH 9.6).

2.5.2 Washing buffer

Washing buffer was prepared by addition of 8.00 g NaCl (Sigma), 0.20 g KH_2PO_4 (Fisons), 1.15 g Na_2HPO_4 (BDH), 0.20 g KCl (BDH), 1 g bovine serum albumin (BSA) (Sigma) and 0.5 ml Tween-20 (BDH) to 1 litre of distilled water.

2.5.3 Blocking buffer

Blocking buffer was prepared by addition of 1% (w/v) BSA to sterile PBS.

2.5.4 Phosphate citrate buffer

Phosphate citrate buffer was prepared by addition of 21.00 g citric acid (BDH) and 14.20 g Na_2HPO_4 (BDH) to 1 litre of distilled water (pH 5.0).

2.5.5 Substrate of detection of HRP-labelled antibodies

The substrate contained 40 mg of ortho-phenylenediamine dihydrochloride (OPD) (Sigma) in 100 ml of phosphate citrate buffer and was activated immediately prior to use with 40 µl 30% (v/v) H_2O_2 (Sigma).

2.5.6 Stopping solution

Stopping solution was 12.5% (v/v) H_2SO_4 diluted in distilled water.

2.6 ELISA to detect Lewis^a and Lewis^b antigens in human milk, infant formula and cows' milk

Plates were coated overnight at 4°C with 100 µl of breast milk, infant formula or cows' milk samples diluted 1 in 10, 1 in 100 or 1 in 1,000 in coating buffer. Positive controls of saliva samples from a non-secretor (Lewis^a) and a secretor (Lewis^b) donor and a negative control of a saliva sample from a Lewis negative donor, were also included on each plate. The plates were washed 3 times with washing buffer and 100 µl of blocking buffer were added to each well for 30 min at room temperature. The plates were washed 3 times and 100 µl of mouse monoclonal anti-Lewis^a or anti-Lewis^b (SNBTS) both diluted 1 in 50 in blocking buffer were added to the plates for 2 h at room temperature. The plates were washed 3 times and 100 µl of HRP-anti-mouse IgG (SAPU) diluted 1 in 100 in blocking buffer were added to the plates for 2 h at room temperature. The OPD substrate (2.5.5) was added to the wells, incubated at room temperature for 15 - 30 min and the reaction stopped with the stopping solution (2.5.6). The absorbance at 490 nm (A_{490}) was determined and

the A_{490} for the negative control wells subtracted from the test sample A_{490}. The results for the test samples were compared with those for the non-secretor, secretor and Lewis negative controls.

2.7 Source of staphylococcal and clostridial toxins

Table 2.2 lists the toxins used and their sources.

Table 2.1 List of monoclonal / polyclonal antibodies, recombinant cytokines and their sources

Antibody / Recombinant	*Source*
Mouse monoclonal anti-human IL-6	R&D*
Recombinant human IL-6	R&D*
Goat polyclonal anti-human IL-6	R&D*
Mouse monoclonal anti-human IL-1β	R&D*
Recombinant human IL-1β	R&D*
Goat polyclonal anti-human IL-1β	R&D*
Mouse monoclonal anti-human TNF-β	R&D*
Recombinat human TNF-β	R&D*
Goat polyclonal anti-human TNF-β	R&D*
HRP anti-sheep/goat IgG	SAPU**
HRP anti-mouse IgG	SAPU**
HRP goat anti-human IgA	Sigma
Standard human IgA derived from colostrum	Sigma
FITC-labelled anti-mouse IgG	Sigma
HRP anti-horse IgG	Sigma
Mouse monoclonal anti-Lewis^a	SNBTS***
Mouse monoclonal anti-Lewis^b	SNBTS***
HRP sheep anti-TSST-1	Toxin Technology
HRP sheep anti-SEC	Toxin Technology
C. perfringens anti-toxin	Mr R. Brown, Dept. of Medical Microbiology
Anti-H type 2	Serotec
HRP sheep anti-bovine IgA	Bethyl Laboratories, Universal Biologicals Limited
HRP sheep anti-bovine IgG_1	Bethyl Laboratories, Universal Biologicals Limited
Bovine immunoglobulin reference serum	Bethyl Laboratories, Universal Biologicals Limited

* R & D Systems Europe Ltd., 4-10 The Quadrant, Barton Lane, Abingdon, Oxon, OX14 3YS, UK.
** Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, ML8 5ES, Scotland.
*** Scottish National Blood Transfusion Service, Ellen's Glen Road, Edinburgh, EH17 7QT.

Table 2.2 List of toxins and their sources

Toxin	*Source*
Toxic shock syndrome toxin (TSST-1)	Toxin Technology
Staphylococcal enterotoxin C (SEC)	Toxin Technology
C. perfringens enterotoxin A (CEA) (number 8237)	Dr D. Williamson, The Defence Microbiology Division (CBDE), Porton Down

Chapter Three

The effect of human milk and infant formula preparations on binding of toxigenic *Staphylococcus aureus* to epithelial cells

3.1 Introduction

Many of the risk factors for respiratory tract infections are also associated with SIDS. Breast feeding reduces susceptibility to respiratory tract infections [Pisacane *et al.*, 1994] and oligosaccharide components in human milk have been demonstrated to inhibit binding of the bacterial respiratory pathogens *Streptococcus pneumoniae* and *Haemophilus influenzae* to human pharyngeal or buccal epithelial cells [Andersson *et al.*, 1986].

Prior to death, many SIDS victims had mild symptoms of respiratory tract infections. Compared to healthy infants in the same age range, toxigenic bacteria such as *S. aureus* and *Streptococcus* species are more likely to be identified among SIDS infants [Telford *et al.*, 1989]. In a longitudinal survey of healthy infants in the age range at greatest risk of SIDS, 56% were colonised by *S. aureus* but these bacteria were identified in 86% of 39 local SIDS infants examined during the same period [Blackwell *et al.*, 1999].

The Lewis^a antigen is expressed by 80-90% of infants during the 2 - 4 month age range in which most deaths occur. Lewis^a is a receptor for *S. aureus* [Saadi *et al.*, 1993] and an adhesin has been isolated from these bacteria by affinity purification with synthetic Lewis^a [Saadi *et al.*, 1994]. Epidemiological studies have found the peak in isolation of *S. aureus* from infants in the 2 – 4 month age range parallels expression of the antigen [Aniansson *et al.*, 1992, Blackwell *et al.*, 1999, Harrison *et al.*, 1999a]. Work by other groups has suggested that oligosaccharides and

glycoconjugates present in human milk can act as analogues of epithelial cell receptors that bind bacterial adhesins, thereby reducing the ability of bacteria to colonise epithelial surfaces [Andersson *et al.*, 1986, Goldman, 1993].

The first objective of this study was to compare the ability of human milk and infant formula for their effect on binding of *S. aureus* to epithelial cells. The second objective was to assess the effect of the synthetic Lewis^a and Lewis^b antigens on bacterial binding. Two different methods were used in this study. The first was an *in vivo* method in which bacteria, cells and the milk or formula were added together and binding allowed to occur. The second was the conventional experimental or *in vitro* approach in which bacteria were initially incubated with the milk or formula, washed and the treated bacteria added to the cells. Previous studies have used the *in vitro* approach [Saadi *et al.*, 1993], but the *in vivo* approach was assessed to try to simulate more closely competitive interactions that occur on mucosal surfaces.

3.2 Material and Methods

3.2.1 Preparation and labelling of S. aureus

3.2.1.1 Collection and numeration of bacteria

S. aureus were grown as described in 2.2 and harvested in PBS using sterile swabs (Greiner). The bacteria were washed twice by centrifugation (2,000 x *g* for 20 min) and resuspended in PBS. The number of bacteria ml^{-1} were determined using the standard curve (Figure 3.1).

3.2.1.2 FITC labelling of bacteria

The washed bacteria were labelled with 2 ml of 0.02% (w/v) fluorescein isothiocyanate (FITC) (Sigma) dissolved in FITC buffer (0.05M sodium carbonate and 0.1M sodium chloride dissolved in distilled water, pH 9.2). After 30 min incubation in the dark at room temperature, the bacteria were washed 3 times with PBS at 2,000 x *g* for 20 min.

3.2.2 Bacterial binding assays

3.2.2.1 In vitro protocol

For the *in vitro* protocol, FITC-labelled bacteria (200 µl) were incubated for 1 h at 37°C in an orbital incubator (100 rpm) with breast milk, infant formula or the synthetic Lewis antigens, washed twice and added to an equal volume of Kato III cells at various ratios of bacteria per cell. After incubation for 1 h at 37°C, the cells were washed twice by centrifugation at 300 x *g* for 10 min with PBS and fixed with

1% (v/v) buffered paraformaldehyde. The results were analysed with an EPICS XL flow cytometer (Coulter) as described in 3.2.7.

3.2.2.2 In vivo protocol

For the *in vivo* approach, breast milk, infant formula preparations, synthetic Lewis^a or Lewis^b antigens (Dextra) were mixed with cells and bacteria and the experiment carried out as in 3.2.2.1.

3.2.3 Preparation of the synthetic Lewis antigens

The synthetic Lewis^a and Lewis^b antigens (Dextra) were prepared in sterile PBS and kept at 4°C with 0.05% sodium azide.

3.2.4 Detection of Lewis^a, Lewis^b and H type 2 antigen expression on the surface of bacteria and Kato III cells

Bacteria (2 x 10^8 bacteria ml^{-1}) or Kato III cells (5 x 10^5 cells ml^{-1}) were incubated with 1 in 5, 1 in 10 or 1 in 20 dilutions of murine monoclonal anti-Lewis^a, anti-Lewis^b (SNBTS) or anti-H type 2 (Serotec) in PBS. Incubation was carried out for 30 min at 37°C in an orbital incubator (100 rpm) and the bacteria or cells were washed twice with PBS. FITC-labelled anti-mouse IgM (200 µl) (Sigma) diluted 1 in 20 in PBS was added to all tubes and incubated as above. The bacteria or cells were washed twice and fixed in 1% (v/v) buffered paraformaldehyde. Negative controls of bacteria or cells incubated with PBS alone and bacteria or cells incubated with

FITC-labelled anti-mouse IgM only were included. The results were read by flow cytometry.

3.2.5 Inhibition of bacterial binding by pre-treatment of Kato III cells with anti-Lewis^a or anti-Lewis^b

Kato III cells (5x 10^5 cells ml^{-1}) (200 µl) were incubated with 200 µl of anti-Lewis^a or anti-Lewis^b diluted 1 in 5, 1 in 10 or 1 in 100 in PBS for 60 min at 37°C in an orbital incubator (100 rpm). Cells were washed twice and incubated with 200 µl of FITC-labelled bacteria (400 per cell) for 60 min at 37°C in an orbital incubator (100 rpm). Cells were washed twice and fixed in 1% (v/v) buffered paraformaldehyde. Negative controls of cells incubated with PBS alone or cells incubated with anti-Lewis^a or anti-Lewis^b were included. The results were read by flow cytometry.

3.2.6 Binding of biotinylated blood group antigens to whole bacteria

Bacteria were washed twice and half of the suspension heated to 100°C for 60 min to inactivate bacterial peroxidases. After washing, both heat-treated and control bacteria were resuspended in coating buffer at dilutions of 1 x 10^7, 1 x 10^8 and 1 x 10^9 bacteria ml^{-1} and 96-well flat-bottomed microtitre plates were coated with 100 µl of the suspensions overnight at 4°C. The plates were washed 3 times with washing buffer and 100 µl of the biotinylated antigens Lewis^a, Lewis^b, H type 2, A or B (diluted to 10 µg ml^{-1} in blocking buffer) (Syntesome) were added and incubated at 37°C for 60 min. The antigens were removed and the plates washed 3 times. Streptavidin-HRP (100 µl, diluted to 1.3 µg ml^{-1} in blocking buffer) (Sigma)

were added to the plates and incubated at 37°C for 60 min. The OPD substrate was added and the colour change stopped after approximately 10 min. The A_{490} was recorded using an ELISA plate reader (Dynatech). Controls of heat-treated and control *Helicobacter pylori* 11637 (3.2×10^8 bacteria ml^{-1}) which has adhesins that bind Lewis^a, Lewis^b and H type 2 and antibodies against the different blood group antigens were included as positive controls. Negative controls were bacteria to which no synthetic antigens were added but to which the streptavidin-HRP and substrate were added.

3.2.7 EPICS XL flow cytometer

The binding study results were analysed with an EPICS XL flow cytometer (Coulter). Cells were selected from a display of forward angle light scatter versus 90° light scatter by means of a bitmap. The percentage of cells with fluorescence channel values greater than the controls (cells alone) were obtained from a one-parameter histogram measuring fluorescence on a logarithmic scale. The mean fluorescence channel values for positive cells were also obtained from a one-parameter histogram measuring fluorescence on a logarithmic scale. The results were expressed as the binding index (BI) of each sample calculated by multiplying the percentage of fluorescent cells by the mean fluorescence channel value.

3.2.8 Statistical methods

For comparison of inhibition of binding of bacteria by blocking cell receptors with antibodies to Lewis^a or Lewis^b or blocking the bacterial adhesin with the synthetic

Lewis^{a} or Lewis^{b} antigens, the BI of cells in each sample was compared with the control by the formula: % inhibition = 100 – [(BI of the sample / BI of the control) x 100]. The results were assessed by Student's t-test. For the effect of whole and defatted breast milk or infant formula on bacterial binding to epithelial cells, the control of cells with bacteria and PBS was given a value of 100% and all other values were expressed as a percentage of the control. Those results with a percentage value below 100 demonstrated inhibition of binding whilst those values greater than 100 demonstrated enhanced binding. The results were compared between dilutions: 1 in 10 and 1 in 100; 1 in 100 and 1 in 1,000; 1 in 1,000 and 1 in 10,000. The results for the *in vivo* and *in vitro* methods were compared for each dilution by Student's t-test for paired samples.

3.3 Results

3.3.1 Standard curves for total bacterial count

The standard curve of optical density in relation to the total number of bacteria ml^{-1} for *S. aureus* (Figure 3.1) was used in all subsequent binding studies.

3.3.2 Effect of FITC-labelling on bacterial binding

Bacteria were washed and half the preparation was labelled with FITC and half were unlabelled. Both labelled and unlabelled bacteria were diluted to ratios of 100 and 200 bacteria per cell and 200 µl of the appropriate bacterial suspension were added to 200 µl of Kato III cells (5×10^5 cells ml^{-1}). Binding was allowed to occur for 1 h at 37°C in an orbital incubator (100 rpm). The cells were washed twice in PBS at 300 x *g* for 10 min and fixed in 1% (v/v) buffered paraformaldehyde.

A smear of each suspension was made on a microscope slide and Gram stained to detect bound bacteria which were counted by light microscopy (100 x). A control of cells alone was included. For each ratio of bacteria : cell, the numbers of bacteria per cell were counted for 20 cells and the mean of 2 individual slides are shown in Figure 3.2. As with previous studies comparing binding of FITC-labelled and unlabelled bacteria (*S. aureus*, *H. pylori*, pneumococci), the results indicate that FITC-labelling does not significantly enhance or reduce binding of *S. aureus* to the epithelial cell line used.

Figure 3.1 OD (540 nm) in relation to the total number of bacteria ml^{-1} determined by microscopy

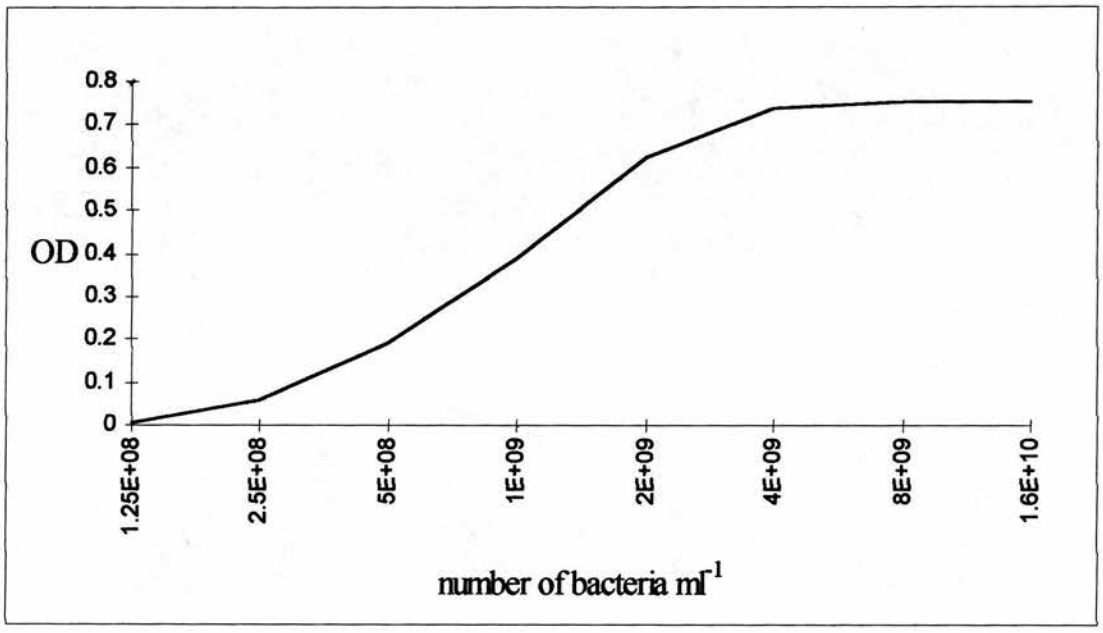
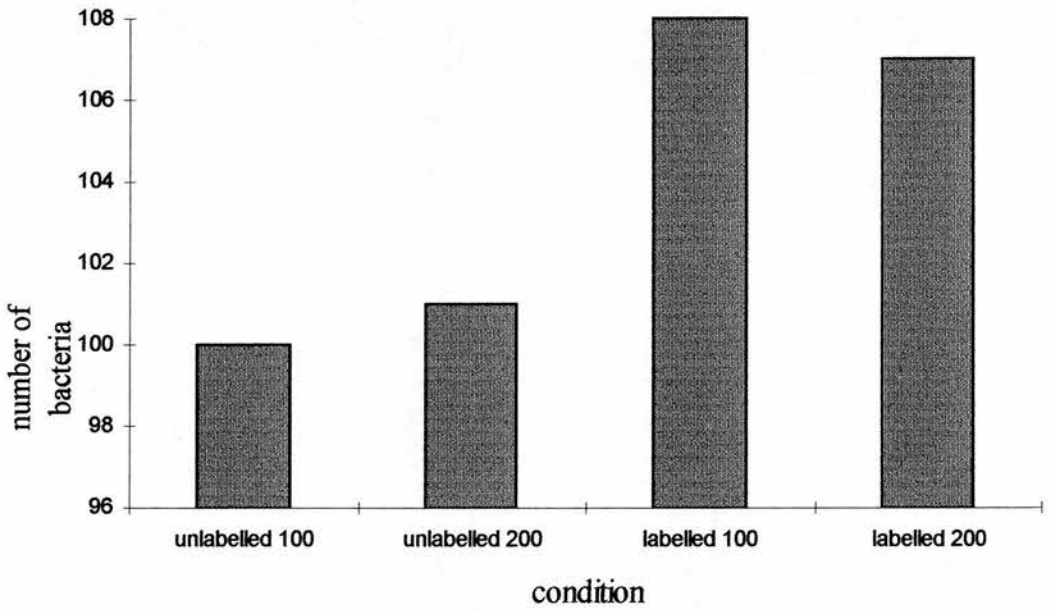


Figure 3.2 The effect of FITC-labelling on binding of *S. aureus* to Kato III cells



3.3.3 Optimisation of incubation time and ratio of bacteria to Kato III cells

FITC-labelled *S. aureus* was incubated for 15, 30, 60 and 120 min at five different ratios of bacteria per cell: 85, 170, 340, 680 and 1360. The results are presented in Figures 3.3a and 3.3b. The binding index (BI) did not appear to increase significantly with increasing incubation after 15 min. For binding assays, an incubation time of 60 min was chosen and a ratio near the mid-point of the linear curve was chosen (400 bacteria per cell) to detect differences due to treatment with different reagents.

3.3.4 Detection of Lewis^a, Lewis^b and H type 2 antigen expression on the surface of Kato III cells and S. aureus

Binding of monoclonal antibodies was assessed to determine if Kato III cells express these antigens on their surface. Binding of the antibodies to *S. aureus* was also assessed as many species have surface components cross-reactive with blood group antigens. The results are shown in Table 3.1 and represent the mean binding indices from three experiments.

The Kato III cells bound high levels of anti-Lewis^a and anti-Lewis^b but lower levels of anti-H type 2. Both Lewis^a and Lewis^b can be used as receptors for *S. aureus*. The bacteria also bound the monoclonal antibodies with anti-Lewis^b exhibiting the highest binding index and anti-H type 2 the lowest.

Figure 3.3a Effect of number of bacteria per cell on binding of *S. aureus* to Kato III cells

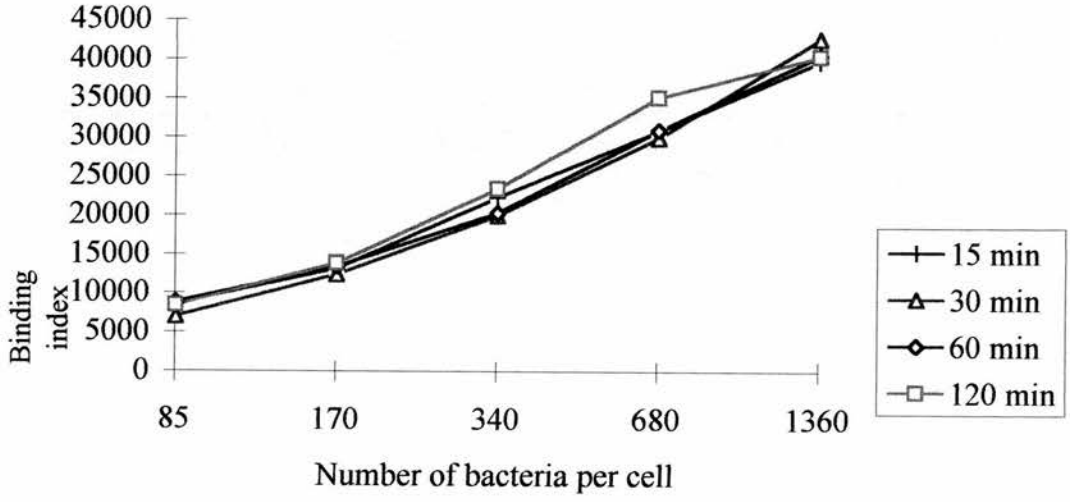


Figure 3.3b Effect of time on binding of *S. aureus* to Kato III cells

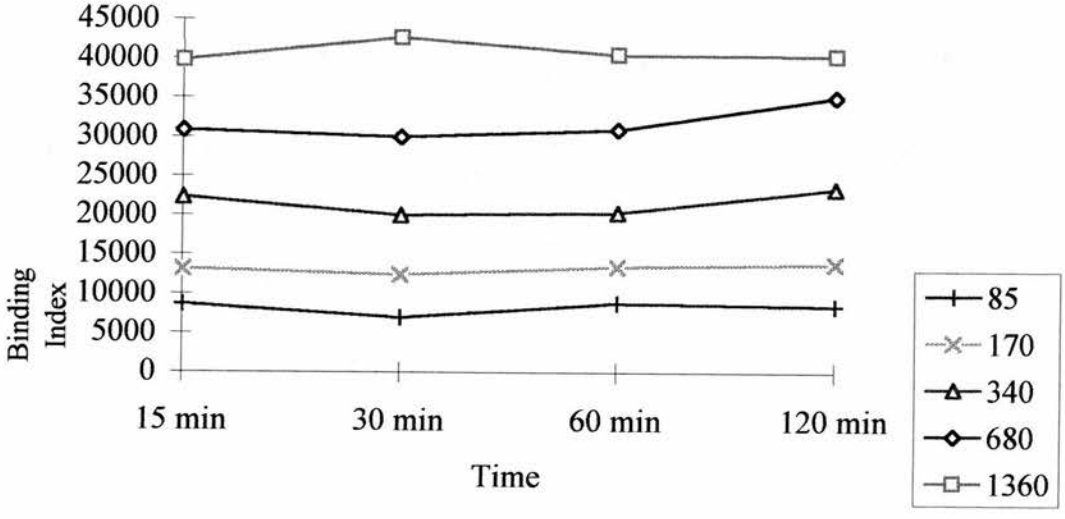


Table 3.1 Mean binding indices obtained with monoclonal antibodies to Lewis[a], Lewis[b] or H type 2 binding to a) Kato III cells and b) *S. aureus* (3 experiments)

a) Kato III cells

Monoclonal Antibody Dilution	Cells Alone	Cells plus FITC anti-mouse IgM	Anti-Lewis[a]	Anti-Lewis[b]	Anti-H type 2
1 in 5	491	873	41,615	33,710	9,139
1 in 10	491	873	36,849	30,950	3,685
1 in 20	491	873	30,276	30,848	2,684

b) *S. aureus*

Monoclonal Antibody Dilution	Bacteria alone	Bacteria plus FITC anti-mouse IgM	Anti-Lewis[a]	Anti-Lewis[b]	Anti-H type 2
1 in 5	238	593	35,223	39,495	8,830
1 in 10	238	593	22,746	35,128	4,712
1 in 20	238	593	13,383	28,804	3,349

3.3.5 Inhibition of bacterial binding by pre-treatment of Kato III cells with anti-Lewis^a or anti-Lewis^b

Both anti-Lewis^a and anti-Lewis^b inhibited binding of *S. aureus* (Table 3.2). This indicates that *S. aureus* can use both Lewis^a and Lewis^b antigens expressed on the surface of Kato III cells as receptors. Although anti-Lewis^b appeared to bind in greater levels to the bacteria, similar inhibition was obtained by pre-treatment of the Kato III cells with the two antibodies.

3.3.6 Binding of biotinylated blood group antigens to whole bacteria

It was found that heat-treated bacteria provided more reliable results than untreated bacteria which exhibited colour changes with substrate alone. This can be explained by the presence of peroxidases which react with the substrate. Heating at 100°C denatures these enzymes but not the adhesins that bind Lewis antigens [Alkout *et al.*, 1997].

Adhesins that bind Lewis^a have been isolated by affinity purification from outer membrane preparations of *S. aureus* and *H. pylori* [Saadi *et al.*, 1994; Alkout *et al.*, 1997]. The results for binding of biotinylated blood group antigens are shown in Table 3.3a (for *S. aureus*) and 3.3b (for *H. pylori*). This experiment provided direct evidence that there are adhesins on *S. aureus* that bind the Lewis^a and Lewis^b antigens. There are also components that can bind H, A and B blood group antigens.

Table 3.2 Mean percentage inhibition of binding of *S. aureus* following pre-treatment of Kato III cells with monoclonal anti-Lewis[a] or anti-Lewis[b] (mean of 2 experiments)

Condition	Binding Index Anti-Lewis[a]	% inhibition	Binding Index Anti-Lewis[b]	% inhibition
cells alone plus PBS	388	-	388	-
cells plus anti-Lewis	590	-	436	-
cells plus *S. aureus*	18,092	-	18,092	-
monoclonal antibody dilution 1 in 5	9,515	47.4	8,672	52.1
monoclonal antibody dilution 1 in 10	9,101	49.7	10,743	40.6
monoclonal antibody dilution 1 in 20	12,172	32.7	10,832	40.1

Table 3.3 a) Binding of biotinylated blood group antigens to *S. aureus* (mean of 6 experiments)

Blood group antigen	1 x 10^7 bacteria ml^{-1} (A_{490})	1 x 10^8 bacteria ml^{-1} (A_{490})	1 x 10^9 bacteria ml^{-1} (A_{490})
Lewis[a]	0.070	0.088	0.138
Lewis[b]	0.061	0.128	0.120
H	0.113	0.104	0.115
A	0.125	0.162	0.178
B	0.062	0.077	0.106
Bacteria alone	0.024	0.042	0.065

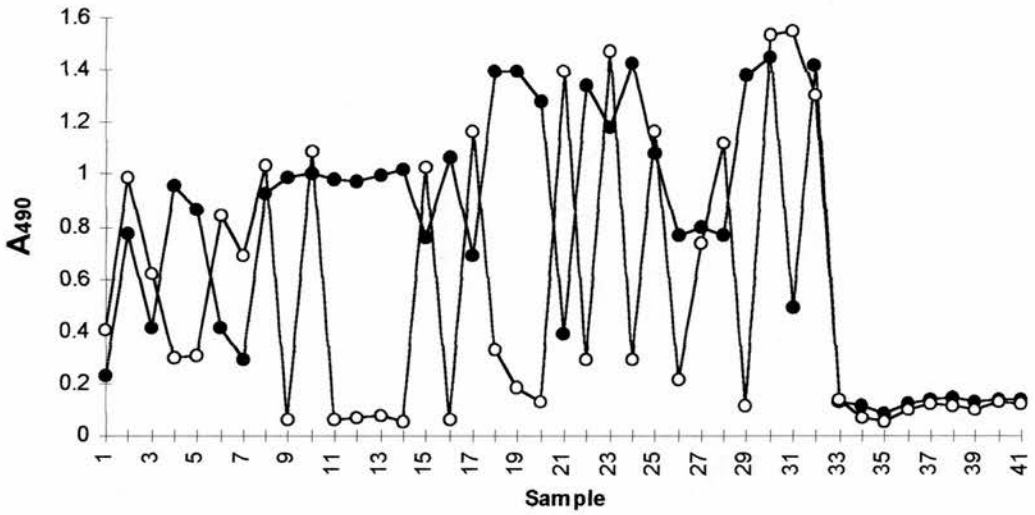
Table 3.3 b) Binding of biotinylated blood group antigens to *H. pylori* (mean of 6 experiments expressed as A_{490})

Blood group antigen	3.2 x 10^8 bacteria ml^{-1} (A_{490})
Lewis[a]	0.114
Lewis[b]	0.119
H	0.220
A	0.206
B	0.122
Bacteria alone	0.026

3.3.7 Detection of Lewis^a and Lewis^b in human milk, cows' milk and infant formula preparations

At a dilution of 1 in 100, the 32 human milk samples bound monoclonal antibodies to Lewis^a and Lewis^b antigens (Figure 3.4). The levels detected varied among donors depending on secretor status; women who were non-secretors (samples 9, 11, 12, 13, 14, 16, 19, 20, 29) had very low readings for Lewis^b. In contrast to the human milk samples, there were very low levels of binding of monoclonal anti-Lewis^a and anti-Lewis^b for whole, semi-skimmed, skimmed or organic cows' milk (samples 33 to 36) at the 1 in 100 dilution. Low readings were also obtained with the infant formulas samples 37 to 41 at the 1 in 100 dilution.

Figure 3.4 Binding of monoclonal anti-Lewis[a] (●) or anti-Lewis[b] (○) to individual milk samples (1 to 32), whole, semi-skimmed, skimmed and organic cows' milk (33 to 36) and formula preparations (37 to 41) (each diluted 1 in 100)



3.3.8 Effect of whole and defatted human milk on binding of S. aureus to Kato III cells

Dilutions of milk ranging from 1 in 10 to 1 in 10,000 were tested for their effect on bacterial binding. No significant differences in binding were observed between the 1 in 10,000 dilution and the control of bacteria, cells and PBS; therefore, these dilutions were omitted from the analyses.

In 6 experiments, whole and defatted human milk caused enhancement of bacterial binding with both methods (Tables 3.4a and 3.4b, Figure 3.5). Greater enhancement of binding was observed for the *in vitro* method compared with the *in vivo* method, and this enhancement effect was reduced with increasing dilutions of human milk. Significant enhancement of binding was observed for the *in vivo* method with whole milk at the 1 in 100 dilution ($p=0.016$) and for the *in vitro* method with both whole milk ($p=0.011$) and defatted milk ($p=0.015$) at the 1 in 10 dilution.

Paired sample t-tests were carried out to test for significant differences between different dilutions of whole or defatted milk within the two methods. For the *in vivo* method, significant differences were found for whole milk between dilutions 1 in 100 and 1 in 1,000 ($p=0.008$) and dilutions 1 in 1,000 and 1 in 10,000 ($p=0.034$). For defatted milk, significant differences were only found between dilutions 1 in 10 and 1 in 100 ($p=0.006$). For the *in vitro* method, significant differences were found for whole milk between dilutions 1 in 10 and 1 in 100 ($p=0.041$) and for defatted milk between dilutions 1 in 100 and 1 in 1,000 ($p=0.010$).

Paired sample t-tests were carried out to test for significant differences between the methods for the same dilution. For whole milk, the increase in binding was significantly higher with the *in vivo* method for the 1 in 100 dilution ($p=0.003$) and the 1 in 1,000 dilution ($p=0.004$), but for defatted milk no significant differences between the two methods were observed for any dilution tested. Bacteria incubated with whole milk showed significantly higher binding than those incubated with defatted milk with the *in vivo* method at the 1 in 100 dilution ($p=0.011$) and the 1 in 1,000 dilution ($p=0.024$) but none were observed for the *in vitro* method.

Table 3.4 Effect of whole (W) and defatted (D) breast milk on binding of *S. aureus* to Kato III cells using a) *in vivo* and b) *in vitro* methods (mean of 6 experiments expressed as a percentage of control bacteria treated with PBS and given a value of 100%)

a) in vivo

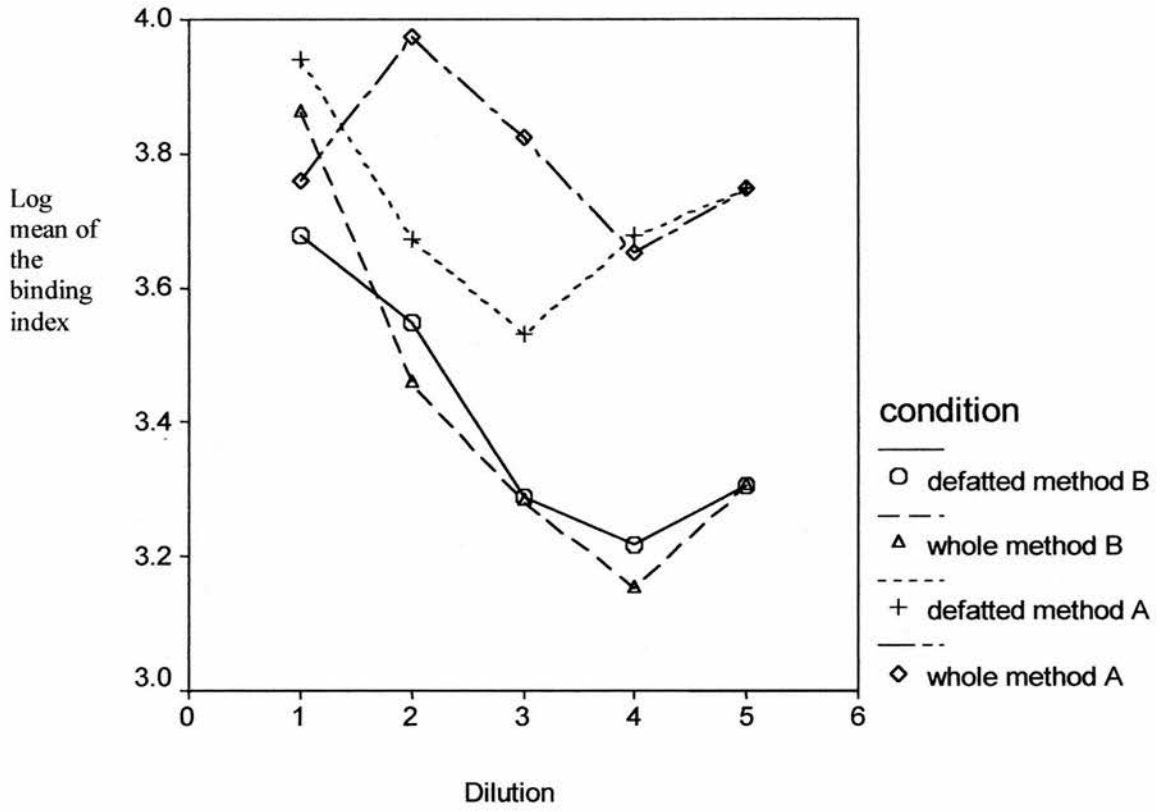
Dilution	%	Se	95% CI	p
1 in 10 W	127.6	31.4	47.0 – 208.3	0.418
1 in 100W	176.7	21.3	121.9 – 231.5	0.016*
1 in 1,000 W	124.3	15.1	85.5 – 163.1	0.168
1 in 10 D	172.9	36.4	79.2 – 266.6	0.102
1 in 100 D	90.7	14.9	52.3 – 129.0	0.560
1 in 1,000 D	67.4	13.1	33.8 – 101.1	0.055

b) in vitro

Dilution	%	Se	95% CI	p
1 in 10 W	409.5	77.8	209.4 – 609.6	0.011*
1 in 100 W	154.1	25.8	87.7 – 220.5	0.090
1 in 1,000 W	108.4	26.4	40.4 – 176.4	0.765
1 in 10 D	258.3	43.4	146.8 – 369.9	0.015*
1 in 100 D	192.0	41.5	85.4 – 298.6	0.077
1 in 1,000 D	110.7	22.6	52.7 – 168.7	0.656

* significant differences from control treated with PBS only

Figure 3.5 Comparison of the *in vivo* (method A) and *in vitro* (method B) methods for the effect of dilution of human milk samples on log mean of the binding index for *S. aureus*



3.3.9 Effects of infant formula preparations on binding of S. aureus to Kato III cells

Three different infant formula preparations were investigated for their effect on binding of *S. aureus* to Kato III cells with both methods. There was no significant variation associated with formula type with either method; therefore, the results obtained for each method and for each dilution have been grouped together (Tables 3.5a and 3.5b, Figure 3.6). In contrast to whole and defatted human milk, the results obtained differed considerably. For the *in vivo* method, infant formula inhibited bacterial binding to Kato III cells and this inhibition of binding was reduced with increasing dilutions of the formula. In contrast, with the *in vitro* method, infant formula caused enhanced bacterial binding to the cells and this enhancement was also reduced with increasing dilutions of the formula.

A significant reduction of binding was observed for the *in vivo* method at the 1 in 10 ($p=0.000$) and 1 in 100 ($p=0.000$) dilutions. A significant enhancement of binding was observed for the *in vitro* method with all three dilutions: 1 in 10, $p=0.004$; 1 in 100, $p=0.001$; 1 in 1,000, $p=0.000$. Significant differences between the *in vivo* and *in vitro* methods for the same dilution were found for the 1 in 10 dilution ($p=0.024$) and the 1 in 100 dilution ($p=0.005$). Significant differences between dilutions were found for the *in vivo* method between dilutions 1 in 100 and 1 in 1,000 ($p=0.003$) and for the *in vitro* method between dilutions 1 in 1,000 and 1 in 10,000 ($p=0.000$).

Table 3.5 Effect of infant formula preparations on binding of *S. aureus* to Kato III cells using a) *in vivo* and b) *in vitro* method (mean of 13 and 12 experiments respectively) expressed as a percentage of results for control bacteria treated with PBS and given a value of 100%)

a) *in vivo*

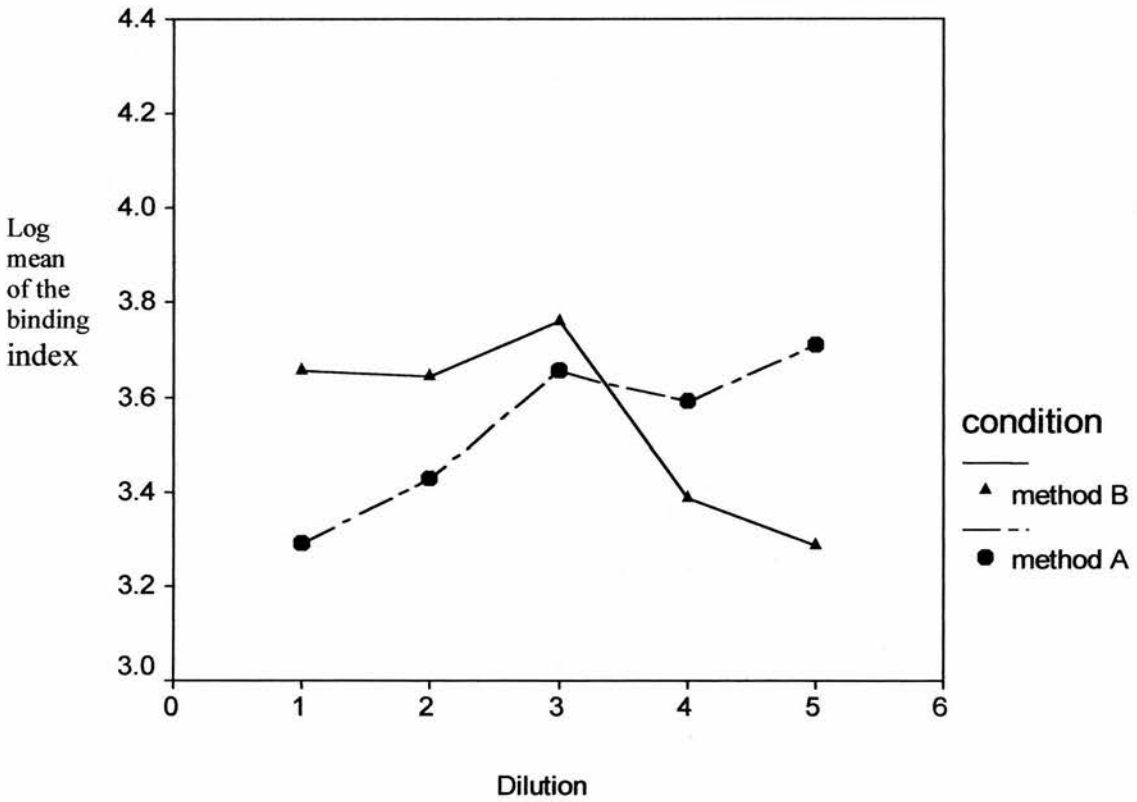
Dilution	%	Se	95% CI	p
1 in 10	44.2	5.9	31.2 – 57.1	0.000*
1 in 100	55.2	4.9	44.6 – 65.8	0.000*
1 in 1,000	90.5	6.3	76.6 – 104.3	0.160

b) *in vitro*

Dilution	%	Se	95% CI	p
1 in 10	288.8	51.0	176.5 – 401.1	0.004*
1 in 100	255.6	34.5	179.7 – 331.4	0.001*
1 in 1,000	325.6	37.8	242.4 – 408.8	0.000*

* significant differences from controls treated with PBS only

Figure 3.6 Comparison of the *in vivo* (method A) and *in vitro* (method B) methods for the effect of dilution of infant formula samples on log mean of the binding index for *S. aureus*



3.3.10 Investigation of enhancement of S. aureus binding

To investigate the enhancement effect, flow cytometry and microscopy studies were carried out and the results from the former are shown in Figure 3.7. The observed enhancement was found to be due to formation of bacterial aggregates by pre-incubation of the bacteria with milk or infant formula.

3.3.11 Inhibition of binding of S. aureus by synthetic Lewis^a or Lewis^b antigens

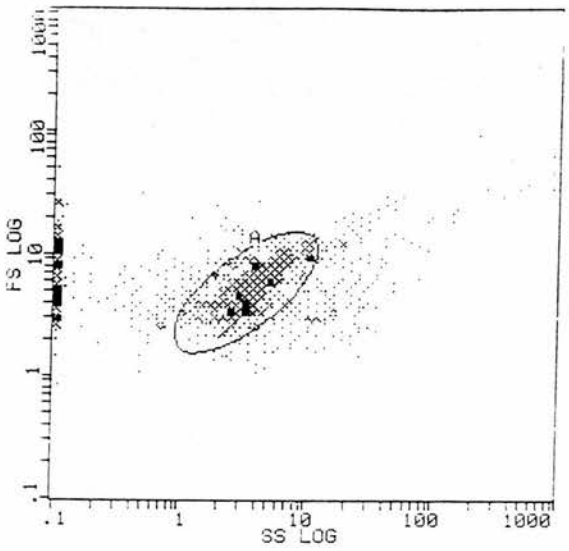
Dose response experiments with synthetic Lewis^a or Lewis^b antigens were carried out with both methods. The dose response curves from 3 experiments are shown as the mean percentage inhibition of binding (Figures 3.8a and 3.8b) and the error bars represent the standard error of the mean (SEM).

Different results were obtained with the two methods for inhibition of binding by the synthetic antigens. Higher levels of inhibition were obtained for Lewis^b with the *in vivo* method whilst higher levels of inhibition were obtained for Lewis^a with the *in vitro* method. Significant inhibition of binding was observed with lower concentrations of Lewis^b compared to Lewis^a. With the *in vivo* method, consistently significant inhibition of binding was observed for Lewis^a between 20 and 40 μg ml^{-1} 36.5 % (95% CI 5.6 – 67.3, p=0.037) and 33.6 % (95% CI 10.6 – 56.5, p=0.024) respectively with a plateau of inhibition reached at 20 μg ml^{-1}. For Lewis^b, significant inhibition of binding was observed between 4 and 5 μg ml^{-1} 43.8% (95% CI 36.9 – 50.8, p=0.008) and 44.4 % (95% CI 34.9 – 54.0, p=0.011) respectively with a plateau of inhibition reached at 20 μg ml^{-1} 53.3% (95% CI 45.1 – 61.6,

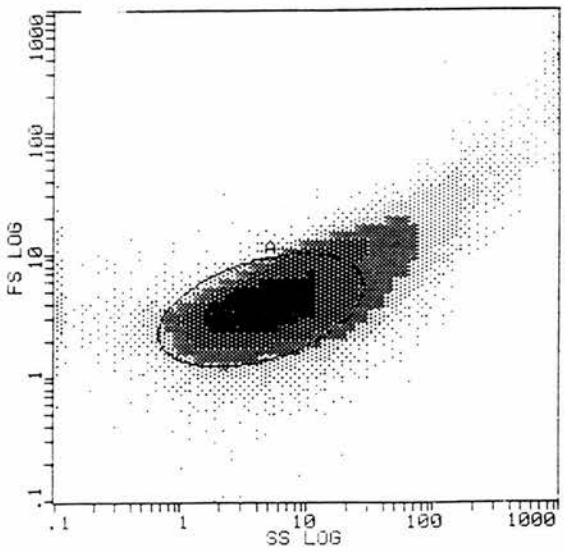
p=0.008). With the *in vitro* method, consistent and significant inhibition of binding was observed for Lewis^{a} between 8 and 10 μg ml^{-1} 47.3% (95% CI 15.5- 79.1, p=0.034) and 56.2% (95% CI 29.7 – 82.7, p=0.012) respectively with a plateau of inhibition reached at 10 μg ml^{-1}. For Lewis^{b}, consistent and significant inhibition of binding was observed between 4 and 6 μg ml^{-1} 42.0% (95% CI 7.1 – 76.8, p=0.035) and 54.7% (95% CI 21.0 – 88.4, p=0.020) respectively, with a plateau reached at 6 μg ml^{-1}.

Figure 3.7 Aggregation of *S. aureus* by pooled whole human milk and infant formula detected by flow cytometry

Bacteria alone



Bacteria plus whole milk
(1 in 10 dilution)



Bacteria plus infant formula
(1 in 10 dilution)

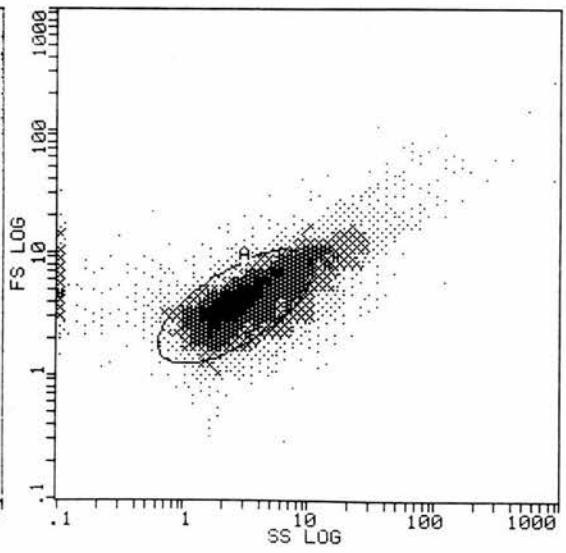
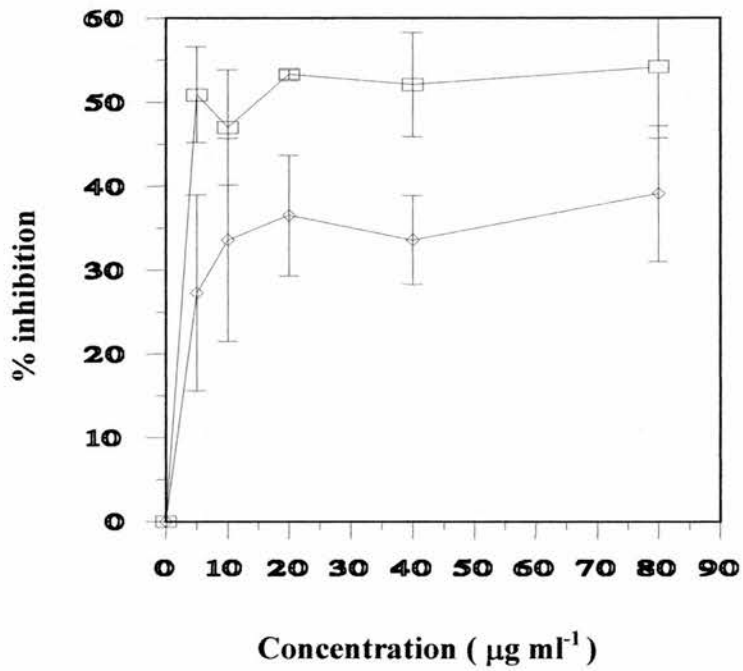
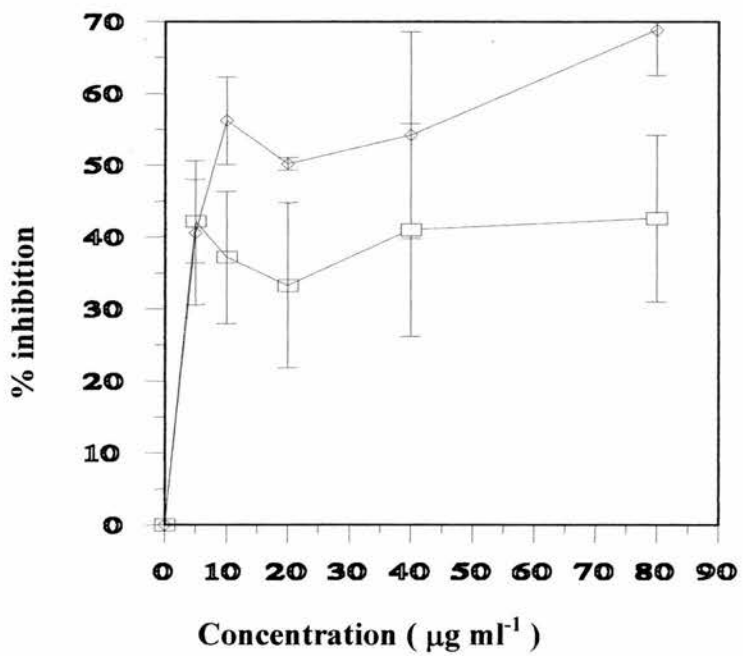


Figure 3.8 Inhibition of binding of *S. aureus* by the synthetic Lewis[a] (diamond) and Lewis[b] (rectangle) antigens (0 – 80 μg ml^{-1}): a) *in vivo* method b) *in vitro* method

a)



b)



3.3.12 Addition of synthetic Lewis^a or Lewis^b antigens to infant formula

Synthetic Lewis^a or Lewis^b antigens were added to two different dilutions of infant formula (1 in 10 and 1 in 100) to give a final concentration of 20 μg ml^{-1}. This concentration was chosen since maximum inhibition of binding was observed with this concentration for the *in vivo* method using the synthetic Lewis antigens (3.3.11). These preparations were used in the *in vivo* system to investigate the potential for improving the inhibitory activity of infant formula against *S. aureus* binding to epithelial cells. Positive controls included in these experiments consisted of cells, bacteria and PBS; cells, bacteria and Lewis antigen; cells, bacteria and the appropriate dilution of formula. The test samples contained cells, bacteria, the appropriate dilution of formula with Lewis^a or Lewis^b antigens.

The mean percentage inhibition of binding for 4 experiments are shown in Table 3.6. Although the addition of Lewis^a or Lewis^b to infant formula increased the observed inhibition of binding compared to infant formula alone, significant differences between the control (cells, bacteria and infant formula) and test samples (cells, bacteria, infant formula and the Lewis antigen) were found only for the 1 in 100 dilution of formula with synthetic Lewis^a added ($p=0.036$).

Table 3.6 Inhibition of bacterial binding (*in vivo*) method by infant formula and infant formula containing 20 μg ml^{-1} of synthetic Lewis[a] or Lewis[b]. The p value represents the difference between the equivalent dilutions of infant formula and infant formula with Lewis antigen added (mean of 4 experiments).

Condition	Mean % inhibition of binding
Formula diluted 1 in 10	54.7
Formula diluted 1 in 10 plus Lewis[a]	61.0 (p = 0.255)
Formula diluted 1 in 10 plus Lewis[b]	60.8 (p = 0.452)
Formula diluted 1 in 100	55.8
Formula diluted 1 in 100 plus Lewis[a]	63.0 (p = 0.036)
Formula diluted 1 in 100 plus Lewis[b]	59.3 (p = 0.741)

3.4 Discussion

Staphylococcal toxins have been identified in tissues of SIDS infants [Newbould *et al.*, 1989; Malam *et al.*, 1992; Zorgani *et al.*, 1999] and a significantly higher isolation rate for *S. aureus* among SIDS infants 3 months of age or younger compared with healthy infants has been reported [Blackwell *et al.*, 1999; Harrison *et al.*, 1999a]. The longitudinal epidemiological studies on nasopharyngeal flora of infants found no association between frequency of isolation of *S. aureus* with socioeconomic circumstances of the family or method of feeding [Harrison, 1998; Blackwell *et al.*, 1999]; however, higher density of colonisation by *S. aureus* was connected with exposure of the infant to cigarette smoke [Harrison, 1998]. The objectives of the present study were to determine if human milk was better able to reduce the density of colonisation of epithelial cells by *S. aureus* compared with infant formula and to determine if oligosaccharides which act as receptors for *S. aureus* could inhibit bacterial binding.

3.4.1 Methodology

The epithelial cell line, Kato III, was chosen for use in the model system to minimise differences in expression of H and Lewis antigens noted for individual donors in other studies [Alkout *et al.*, 1997]. Most studies on inhibition of bacterial binding are carried out by the *in vitro* protocol in which the bacteria or the cells are treated with the inhibitory substance, the excess washed away and the binding assays carried out. This study found that there were significant differences in results obtained by the two protocols. The *in vivo* method is thought to reflect more closely

the interactions between mucosal cells, bacteria and the human or formula milk. The *in vitro* method was more useful for identifying components involved in binding.

3.4.2 Effects of human milk and formula on bacterial binding

The effects of whole and defatted human milk on bacterial binding were similar with both methods. Enhancement of binding rather than inhibition was observed and flow cytometry studies suggest that enhanced binding occurs due to the formation of bacterial aggregates. Greater enhancement of binding was observed using the *in vitro* method in which there was more time for bacteria to aggregate before addition to the cells.

Host protection by secretory IgA has been found to occur through agglutination of bacteria and inhibition of their attachment. Studies by Wold *et al.* [cited in Rudloff *et al.*, 1997] found that an interaction could occur between bacterial mannose lectins and IgA receptor oligosaccharides and this interaction could inhibit bacterial binding to colonic epithelial cells.

For infant formula, significantly different results were obtained with the two methods. The *in vivo* method showed consistent inhibition of binding regardless of formula type whilst the *in vitro* method showed enhancement of binding due to bacterial aggregation.

From the results obtained in this part of the study, breast milk can be suggested to have a protective effect due to aggregation of the bacteria. The factors responsible for aggregation were present in both whole and defatted milk. Bacterial aggregates are more readily engulfed by phagocytes than single bacteria which would result in greater bacterial clearance. If the *in vivo* method reflects conditions on the mucosal surface, formula preparations might reduce the numbers of bacteria attaching to the epithelium.

3.4.3 Components contributing to reduction of bacterial binding

There is increasing evidence that oligosaccharides and glycoconjugates present in human milk can reduce the ability of bacteria to colonise epithelial surfaces by acting as receptor analogues and binding to bacterial adhesins [Goldman, 1993]. The bacteria bound the biotinylated Lewis antigens (3.3.6), and a dose response effect was observed for inhibition of binding of *S. aureus* by the synthetic Lewis^{a} and Lewis^{b} antigens (Figures 3.8a and 3.8b). Higher levels of the Lewis antigens were required to cause maximum inhibition of binding using the *in vivo* method compared to the *in vitro* method. With the *in vivo* method, the bacteria can either bind to the Lewis antigen expressed on the cell surface or the Lewis antigen in solution; however, with the *in vitro* method, direct blocking of the bacterial adhesin occurs before addition to the cells. Although a dose response effect was observed for inhibition with the synthetic Lewis antigens, there was no correlation between the levels of these antigens in individual human milk samples with their inhibitory effect [Saadi *et al.*, 1999]. Other workers have shown that free oligosaccharides purified from low molecular weight fractions of human milk can inhibit the ability

of *Streptococcus pneumoniae* and *H. influenzae* to bind human pharyngeal or buccal epithelial cells [Andersson *et al.*, 1986].

Breast milk was found to contain much higher levels of Lewis antigens compared to infant formula or cows' milk. Work has been carried out by another group to compare human milk, bovine milk and infant formula based on bovine milk for their oligosaccharide content. This work found that whilst human milk had a high content (6 g/l) of neutral oligosaccharides and their fucosylated or sialylated derivatives, bovine milk and infant formula contained very low levels [Rudloff *et al.*, 1997]. Based on these results and the inhibition of binding studies involving the Lewis antigens, the following hypothesis was tested: the inhibitory activity of infant formula in the *in vivo* system might be increased by the addition of Lewis antigens to the formula preparation. Significant differences in binding between the effect of formula alone and the effect of formula plus Lewis antigen were observed only for the 1 in 100 dilution with Lewis^a added. It might be that higher concentrations of the Lewis antigens would need to be added before a significant difference in binding is observed.

3.4.4 Conclusions

In contrast to our original hypothesis, human milk did not reduce bacterial binding in experiments carried out by either the *in vivo* or *in vitro* methods. This suggests that aggregation of bacteria might play a major role in the protection afforded by breast feeding. Infant formula was found to have inhibitory activity in the *in vivo*

experiments but enhanced bacterial binding in the *in vitro* ones. These studies indicate that the method used for binding studies needs to be considered in planning experiments. The *in vitro* method is used most widely and is valuable for assessment of individual components for inhibitory activity. The *in vivo* method was developed to assess the complex interactions at mucosal surfaces.

Density of colonisation is an important factor in development of bacterial diseases due to invasion or toxin production [Beachey *et al.*, 1981] and these results suggest that both human milk and infant formula have oligosaccharide components that might reduce binding of *S. aureus* to epithelial cells. The potential for improving the inhibitory activity of infant formula by addition of Lewis antigens is an area for further investigation.

Chapter Four

The effect of human milk and infant formula preparations on binding of *Clostridium perfringens* to epithelial cells

4.1 Introduction

Breast feeding is associated with protection against enteric infections [Buescher, 1994]. Many SIDS infants have symptoms of gastrointestinal infections prior to death and compared to healthy infants in the same age range, toxigenic strains of *Clostridium perfringens* are more likely to be identified in SIDS infants [Lindsay *et al.*, 1994]. A number of studies have found significantly higher levels of *C. perfringens* and their toxins in SIDS cases compared to non-SIDS cases [Lindsay *et al.*, 1993; Murrell *et al.*, 1993], and one study found *C. perfringens* in faeces from 45.4% of SIDS infants compared to 19.6% of healthy babies [Murrell *et al.*, 1993]. This study also found that formula fed SIDS infants had a significantly higher incidence of *C. perfringens* and its enterotoxin in their faeces than breast fed infants [Murrell *et al.*, 1993]. *C. perfringens* enterotoxin A has been identified in body fluids of SIDS infants [Murrell *et al.*, 1987], and because of its superantigenic nature, has been suggested to have a significant role in some SIDS deaths [Lindsay *et al.*, 1994, Lindsay *et al.*, 1996].

Adherence to the intestinal mucosa allows bacteria to resist expulsion by peristaltic clearing mechanisms and is the first stage in development of a gastrointestinal infection. Human milk is unique in its content of free oligosaccharides, glycoproteins and glycolipids. There is evidence that free oligosaccharides and glycoconjugates can act as soluble receptor analogues of intestinal cell surface carbohydrates. These interfere with either bacterial binding, thereby preventing colonisation, or with binding of toxins to their receptors [Goldman, 1993;

Ashkenazi, 1996]. The Lewis antigens are oligosaccharide components of both breast milk and infant formula [Chapter 3].

Breast fed compared to formula fed infants have a very different faecal flora. In breast fed babies, the predominant organisms are bifidobacteria, lactobacilli and staphylococci. In contrast in formula fed infants, the predominant organisms are enterococci, coliforms and bacteroides [Wharton *et al.*, 1994]. Breast fed infants receive protection against gastrointestinal infections and this might be because lactose and oligosaccharides present in breast milk have been found to facilitate growth of the bifidus flora in the gut [Emmett *et al.*, 1997].

The previous chapter summarised investigations on the effect of human milk, infant formula and synthetic Lewis antigens on binding of the respiratory pathogen *S. aureus* which expresses an adhesin that binds the Lewis antigens [Saadi *et al.*, 1994]. The results in Chapter 3 found the method used significantly influenced the results obtained with human milk compared with infant formula. The objective of this chapter was to determine if similar effects were obtained with a gastrointestinal organism implicated in SIDS.

4.2 Materials and methods

4.2.1 Preparation and labelling of C. perfringens

4.2.1.1 Collection and numeration of bacteria

C. perfringens were grown as described in 2.2 and prepared as described in 3.2.1.1.

4.2.1.2 FITC labelling of bacteria

C. perfringens was labelled with FITC as described in 3.2.1.2.

4.2.2 Bacterial binding assays

Bacterial binding assays with *C. perfringens* were carried out as described in 3.2.2.

4.2.3 Preparation of the synthetic Lewis antigens

The Lewis antigens were prepared and stored as described in 3.2.3.

4.2.4 Detection of Lewis^a, Lewis^b and H type 2 antigen expression on the surface of C. perfringens

Expression of Lewis^a, Lewis^b and H type 2 antigens on the surface of *C. perfringens* was assessed as described in 3.2.4.

4.2.5 Inhibition of C. perfringens binding by pre-treatment of Kato III cells with anti-Lewis^a or anti-Lewis^b

Inhibition of *C. perfringens* binding after pre-treatment of Kato III cells with monoclonal anti-Lewis^a or anti-Lewis^b was assessed as described in 3.2.5.

4.2.6 Binding of biotinylated blood group antigens to whole bacteria

The binding of biotinylated blood group antigens to *C. perfringens* was assessed as described in 3.2.6.

4.2.7 EPICS XL flow cytometer

The binding study results were analysed with an EPICS XL flow cytometer (Coulter) as described in 3.2.7.

4.2.8 Statistical methods

The statistical analyses described in 3.2.8 were used for these studies with *C. perfringens*.

4.3 Results

4.3.1 Standard curve for total C. perfringens count

The standard curve of optical density in relation to the total number of bacteria ml^{-1} for *C. perfringens* was used in all subsequent binding studies (Figure 4.1).

4.3.2 Effect of FITC-labelling on bacterial binding

A binding assay was carried out using FITC-labelled and unlabelled *C. perfringens* at ratios of 100 and 200 bacteria per cell to determine if FITC altered binding using the method described in 3.3.2. For each ratio of bacteria : cell, the numbers of bacteria per cell were counted for 20 cells and the mean results of 2 slides are shown in Figure 4.2. As with previous studies of other bacteria using these methods (*S. aureus*, *H. pylori*, pneumococci), the results indicate that FITC-labelling does not significantly enhance or reduce binding of *C. perfringens* to the epithelial cell line used.

4.3.3 Optimisation of incubation time and ratio of bacteria to Kato III cells

FITC-labelled *C. perfringens* was incubated for 15, 30, 60 and 120 min at five different ratios of bacteria per cell: 85, 170, 340, 680 and 1360. The results are presented in Figures 4.3a and 4.3b. The binding index (BI) increased with increasing time. For binding assays, an incubation time of 60 min was chosen and a ratio near the mid-point of the linear part of the curve was chosen (400 bacteria per cell) to detect differences due to treatment with different reagents.

Figure 4.1 OD (540 nm) in relation to the total number of bacteria ml^{-1} determined by microscopy

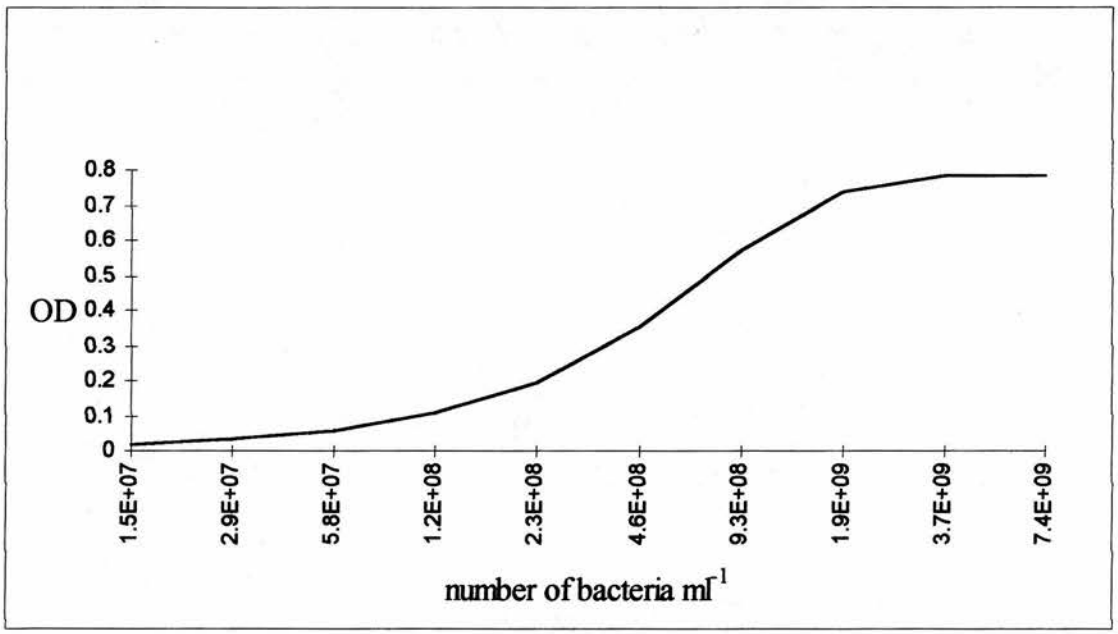


Figure 4.2 The effect of FITC-labelling on binding of *C. perfringens* to Kato III cells (mean of 2 experiments)

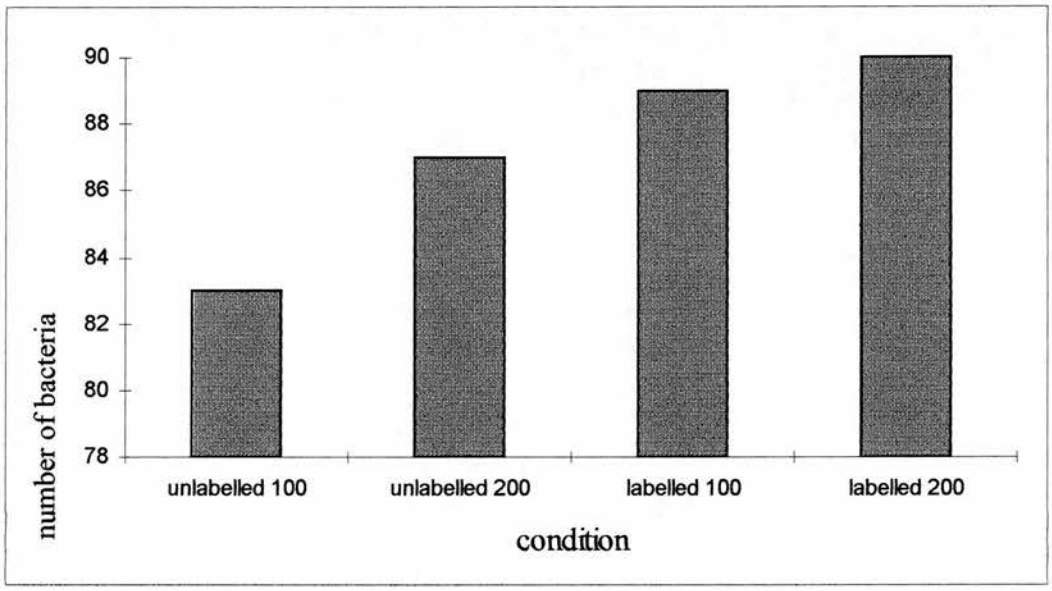


Figure 4.3a Effect of number of bacteria per cell on binding of *C. perfringens* to Kato III cells

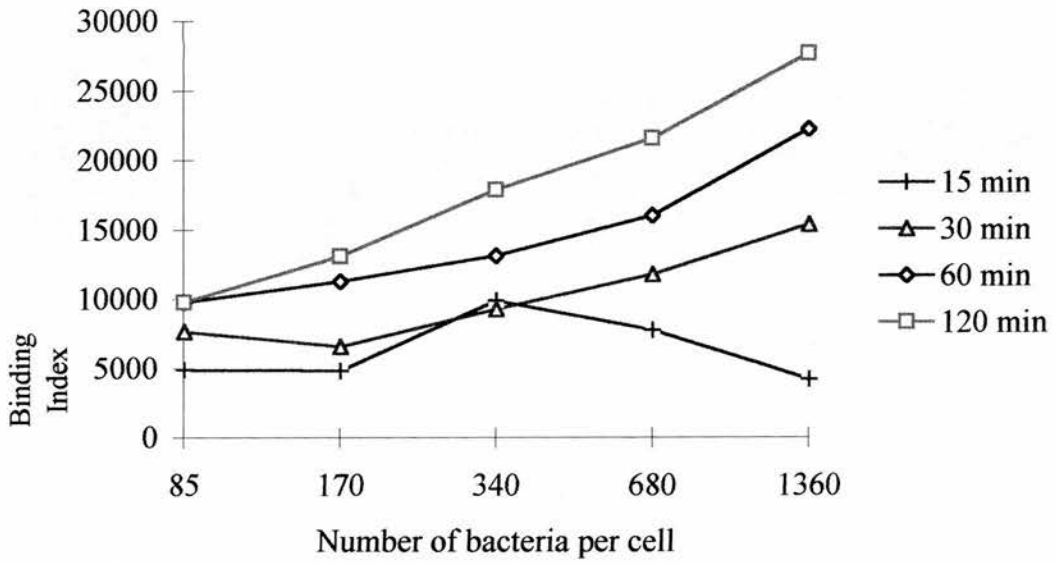
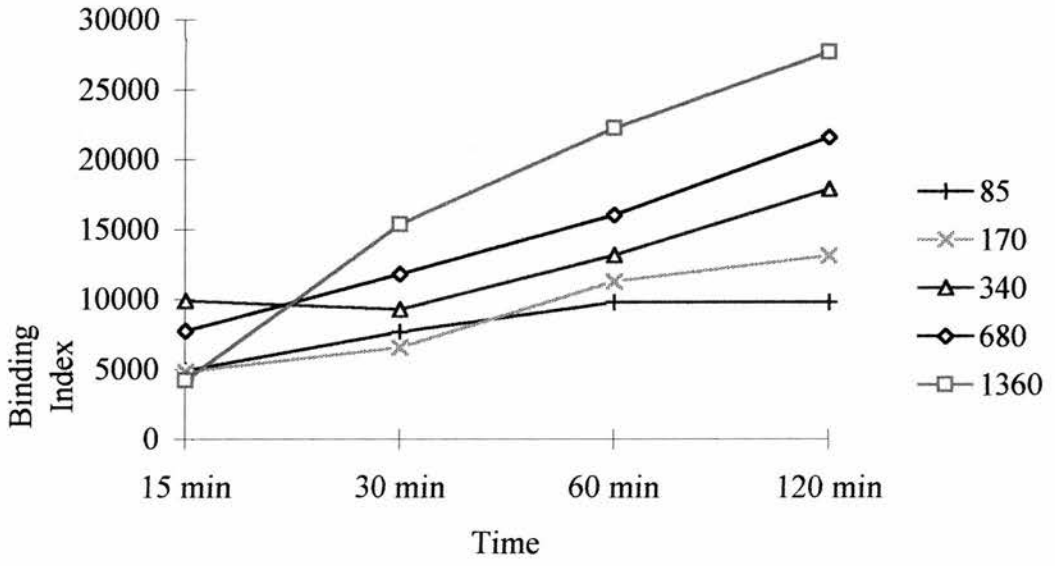


Figure 4.3b Effect of time on binding of *C. perfringens* to Kato III cells



4.3.4 Detection of Lewis^a, Lewis^b and H type 2 antigen expression on the surface of C. perfringens

Binding of monoclonal antibodies specific for Lewis^a, Lewis^b or H type 2 to *C. perfringens* was assessed since many species also have surface components cross-reactive with blood group antigens. The results are shown in Table 4.1 and represent the mean binding index obtained for three experiments. *C. perfringens* had a high binding index for anti-Lewis^a and low binding indices for anti-H type 2 and anti-Lewis^b.

4.3.5 Inhibition of C. perfringens binding by pre-treatment of Kato III cells with anti-Lewis^a or anti-Lewis^b

The percentage inhibition in the binding of *C. perfringens* observed following pre-treatment of Kato III cells with monoclonal antibodies to the Lewis antigens are summarised in Table 4.2. Although inhibition of binding of *C. perfringens* to the antibody coated cells was observed, it did not reflect the differences in binding of the antibodies to the bacteria. The BI for anti-Lewis^a was much higher than that for Lewis^b, but there was little difference in the inhibition studies.

4.3.6 Binding of biotinylated blood group antigens to whole bacteria

The results are shown in Table 4.3 and provide direct evidence that there are adhesins on *C. perfringens* that bind the Lewis^a and Lewis^b antigens as well as the other blood group antigens H, A and B.

Table 4.1 Mean binding indices obtained with monoclonal antibodies to Lewis[a], Lewis[b] or H type 2 binding to *C. perfringens* (3 experiments)

Monoclonal Antibody Dilution	Bacteria alone	Bacteria plus FITC anti-mouse IgM	Anti-Lewis[a]	Anti-Lewis[b]	Anti-H type 2
1 in 5	465	1,031	27,796	3,924	8,760
1 in 10	465	1,031	26,721	3,295	3,807
1 in 100	465	1,031	25,533	2,049	3,006

Table 4.2 Mean percentage inhibition of binding of *C. perfringens* following pre-incubation of Kato III cells with monoclonal anti-Lewis[a] or anti-Lewis[b] (mean of 2 experiments)

Condition	Binding Index Anti-Lewis[a]	% inhibition	Binding Index Anti-Lewis[b]	% inhibition
cells alone plus PBS	388	-	388	-
cells plus anti-Lewis	590	-	436	-
cells plus *C. perfringens*	35,270	-	35,270	-
monoclonal antibody dilution 1 in 5	31,402	11.0	28,020	20.5
monoclonal antibody dilution 1 in 10	30,089	14.7	29,789	15.5
monoclonal antibody dilution 1 in 20	33,032	6.3	30,575	13.3

Table 4.3 Binding of biotinylated blood group antigens to *C. perfringens* (mean of 6 experiments)

Blood group antigen	1 x 10^7 bacteria ml^{-1} (A_{490})	1 x 10^8 bacteria ml^{-1} (A_{490})	1 x 10^9 bacteria ml^{-1} (A_{490})
Lewis[a]	0.093	0.106	0.112
Lewis[b]	0.091	0.098	0.111
H	0.109	0.106	0.175
A	0.198	0.184	0.216
B	0.114	0.077	0.122
Bacteria alone	0.048	0.040	0.064

4.3.7 Effect of whole and defatted human milk on binding of C. perfringens to Kato III cells

Whole and defatted human milk were tested at dilutions of 1 in 10, 1 in 100, 1 in 1,000 and 1 in 10,000. No significant differences in binding were observed between the 1 in 10,000 dilution and the control of bacteria, cells and PBS. The 1 in 10,000 dilution was not used in further analyses.

In 6 experiments, for both methods with whole or defatted human milk, there was a general pattern of enhancement of bacterial binding (Tables 4.4a and 4.4b and Figure 4.4) but the enhancement was not significant compared with the untreated controls. Greater levels of binding were observed with the *in vitro* method, and this was reduced with increasing dilutions of human milk. A small but significant reduction in binding was observed for the defatted milk at higher dilutions.

Paired sample t-tests were carried out to test for significant differences between dilutions of whole or defatted milk within each method. For the *in vivo* method, significant differences were found for whole milk between the 1 in 1,000 and 1 in 10,000 dilutions ($p=0.044$) but no significant differences between dilutions were found for defatted milk. For the *in vitro* method, significant differences between dilutions were found for whole milk between the 1 in 10 and 1 in 100 dilutions ($p=0.009$) and for defatted milk between the 1 in 10 and 1 in 100 dilutions ($p=0.042$).

Paired sample t-tests were carried out to test for significant differences between the two methods for the same dilution. With the *in vitro* method, significantly greater binding was observed for whole milk at the 1 in 100 dilution ($p=0.000$) and the 1 in 1,000 dilution ($p=0.000$) and for defatted milk at each of the three dilutions: 1 in 10 dilution $p=0.001$; 1 in 100 dilution $p=0.000$; 1 in 1,000 dilution $p=0.001$. Paired sample t-tests were also carried out to test for significant differences between whole and defatted milk. Enhancement of binding was significantly higher for whole milk than defatted milk with the *in vivo* method at the 1 in 1,000 dilution ($p=0.045$). With the *in vitro* method, enhancement of binding with whole milk was significantly greater than that for defatted milk at the 1 in 10 dilution ($p=0.026$).

Table 4.4 Effect of whole (W) and defatted (D) breast milk on binding of *C. perfringens* to Kato III cells using a) *in vivo* and b) *in vitro* methods for the mean of 6 experiments expressed as a percentage of control bacteria treated with PBS (100%)

a) *in vivo*

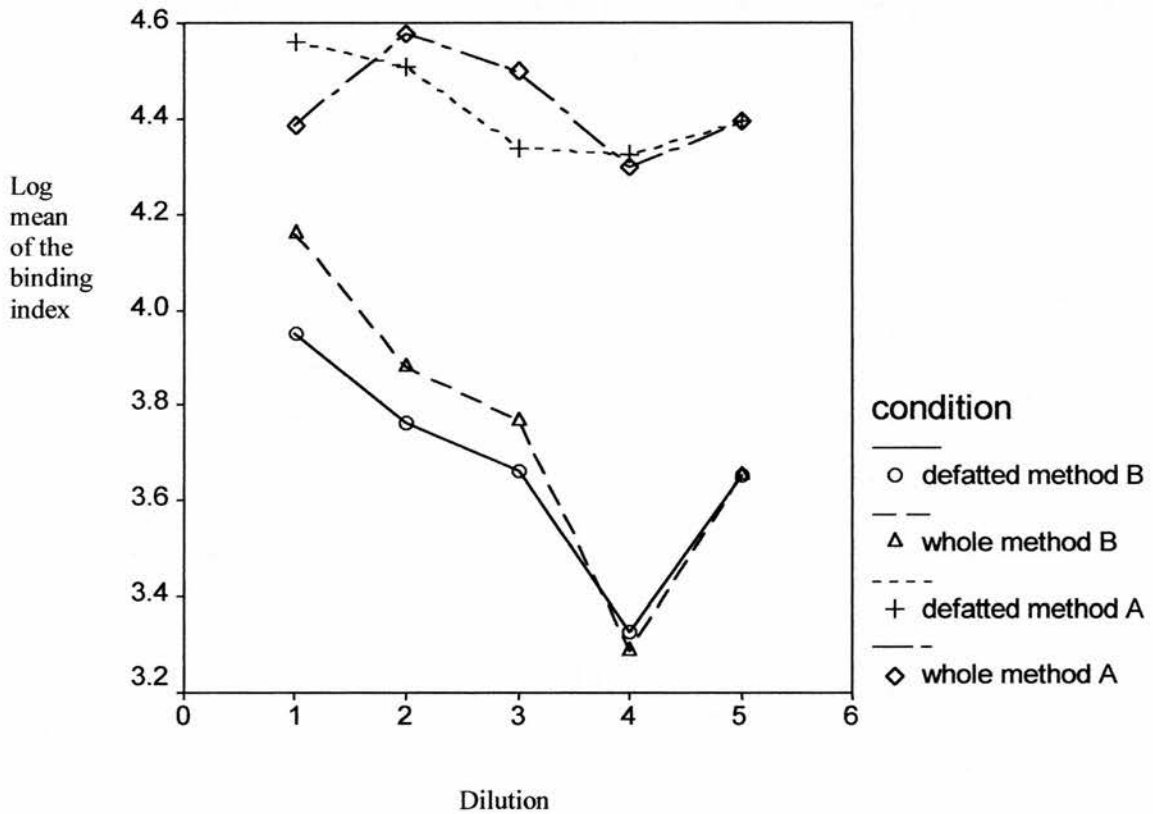
Dilution	%	Se	95% CI	p
1 in 10 W	117.0	29.1	42.1 – 191.9	0.584
1 in 100 W	171.5	34.3	83.2 – 259.8	0.092
1 in 1,000 W	130.7	12.3	99.0 – 162.4	0.055
1 in 10 D	160.3	26.0	93.5 – 227.1	0.068
1 in 100 D	135.3	15.8	94.6 – 176.1	0.076
1 in 1,000 D	88.7	3.4	79.9 – 97.5	0.022*

b) *in vitro*

Dilution	%	Se	95% CI	p
1 in 10 W	512.3	233.1	86.8 – 1111.4	0.137
1 in 100 W	232.1	78.5	30.2 – 433.9	0.153
1 in 1,000 W	167.6	55.9	23.9 – 311.4	0.281
1 in 10 D	245.3	74.2	54.6 – 436.1	0.108
1 in 100 D	147.8	31.2	67.5 – 228.1	0.186
1 in 1,000 D	120.3	30.9	40.9 – 199.7	0.540

** significant differences from controls treated with PBS only*

Figure 4.4 Comparison of the *in vivo* (method A) and *in vitro* (method B) methods for the effect of dilution of human milk samples on log mean of the binding index for *C. perfringens*



4.3.8 Effects of infant formula preparations on binding of C. perfringens to Kato III cells

Three different infant formula preparations were investigated for their effect on binding of *C. perfringens* to Kato III cells using the two methods. When the results from the three different formulas were analysed, it was found that within the methods, there was no significant variation associated with formula type; therefore, the results obtained for each method and for each dilution have been grouped together (Tables 4.5a and 4.5b and Figure 4.5). In contrast to whole and defatted human milk, the results obtained for the *in vivo* and *in vitro* methods differed. With the *in vivo* method, infant formula reduced bacterial binding to Kato III cells at the first two dilutions and this effect was reduced with increasing dilution of the formula. In contrast, with the *in vitro* method, infant formula caused enhanced bacterial binding to the cells and this enhancement was reduced with increasing dilutions of the formula.

A significant reduction in binding was observed for the *in vivo* method for the 1 in 100 ($p=0.023$) dilution and a small but significant increase in binding for the 1 in 1,000 ($p=0.009$) dilution. Significant enhancement of binding was observed for the *in vitro* method for each of the three dilutions: 1 in 10, $p=0.005$; 1 in 100, $p=0.002$; 1 in 1,000, $p=0.005$.

Paired sample t-tests were carried out to test for significant differences between dilutions within each method. Significant differences were found for the *in vivo*

method between dilutions 1 in 100 and 1 in 1,000 (p=0.004) and for the *in vitro* method between dilutions 1 in 100 and 1 in 1,000 (p=0.000) and dilutions 1 in 1,000 and 1 in 10,000 (p=0.000).

Paired sample t-tests were carried out to test for significant differences between the two methods for the same dilutions of infant formula. Significant differences between the two methods for the same dilution were found for *C. perfringens* for all three dilutions: 1 in 10, p=0.020; 1 in 100, p=0.000; 1 in 1,000, p=0.000.

Table 4.5 Effect of infant formula preparations on binding of *C. perfringens* to Kato III cells using a) *in vivo* and b) *in vitro* methods for the mean of 13 and 12 experiments respectively expressed as a percentage of results for control bacteria treated with PBS (100%)

a) *in vivo*

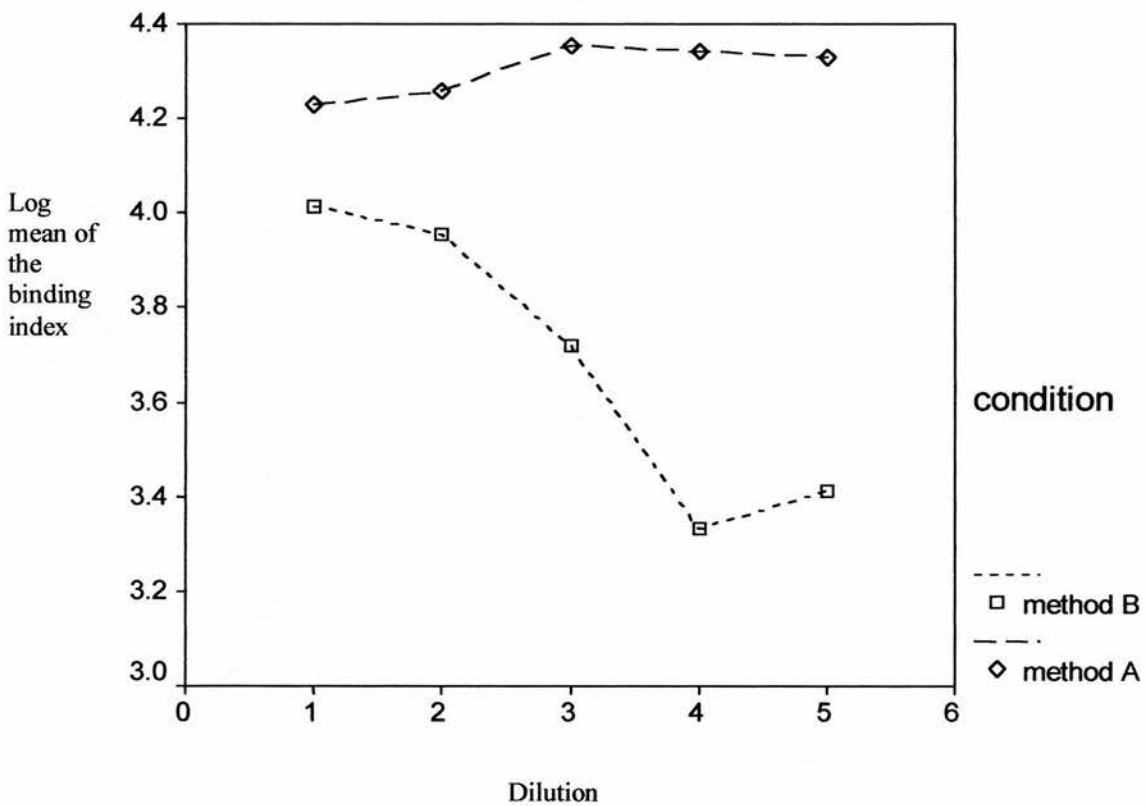
Dilution	%	Se	95% CI	p
1 in 10	85.7	9.5	64.9 – 106.4	0.159
1 in 100	86.6	5.1	75.5 – 97.8	0.023*
1 in 1,000	106.3	2.0	101.9 – 110.8	0.009*

b) *in vitro*

Dilution	%	Se	95% CI	p
1 in 10	559.0	132.2	268.0 – 849.9	0.005*
1 in 100	456.3	90.2	257.7 – 654.8	0.002*
1 in 1,000	241.7	40.2	153.3 – 330.1	0.005*

* significant differences from controls treated with PBS only

Figure 4.5 Comparison of the *in vivo* (method A) and *in vitro* (method B) methods for the effect of dilution on infant formula samples on log mean of the binding index for *C. perfringens*

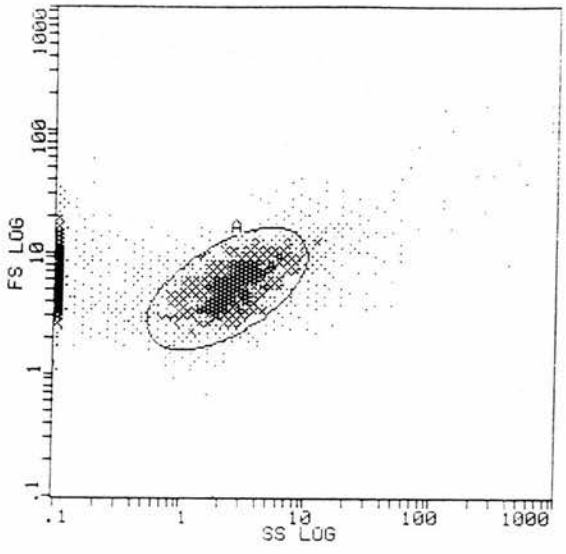


4.3.9 Investigation of enhancement of C. perfringens binding

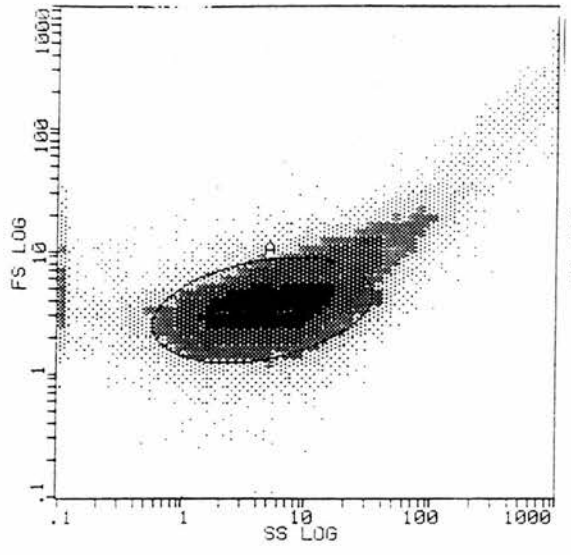
To investigate the enhancement of binding effect, flow cytometry and microscopy studies were carried out and the results of the former are shown in Figure 4.6. The enhancement of binding observed following pre-incubation of the bacteria with either human milk or formula was found to be due to the formation of bacterial aggregates.

Figure 4.6 Aggregation of *C. perfringens* by pooled whole human milk and infant formula detected by flow cytometry

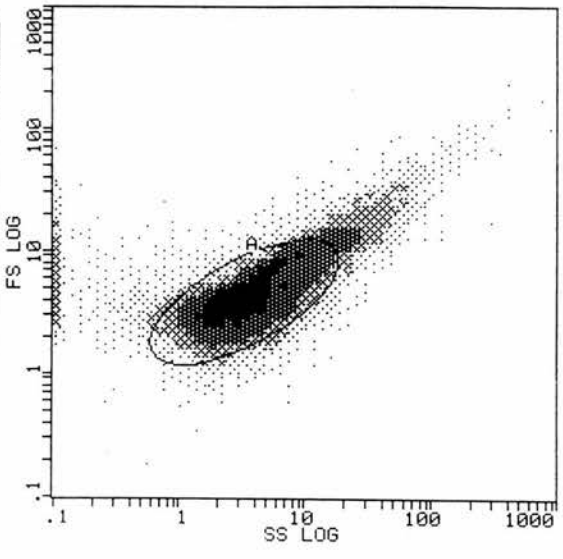
Bacteria alone



Bacteria plus whole milk
(1 in 10 dilution)



Bacteria plus infant formula
(1 in 10 dilution)

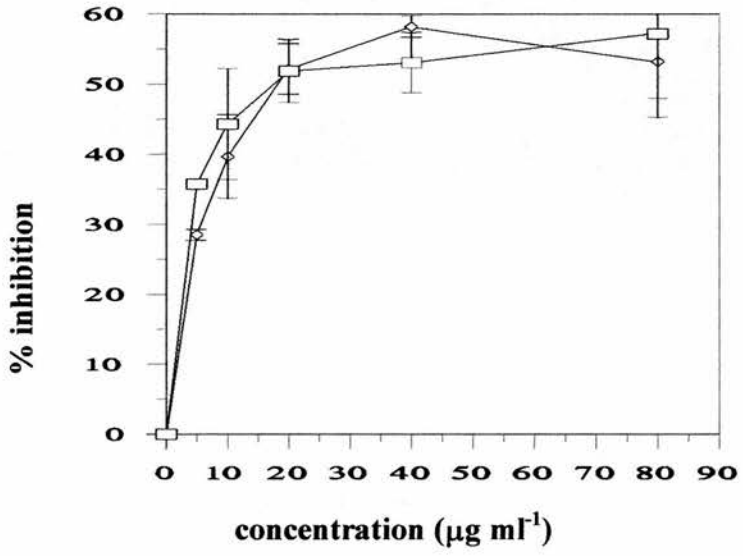


4.3.10 Inhibition of binding of C. perfringens by synthetic Lewis^a or Lewis^b antigens

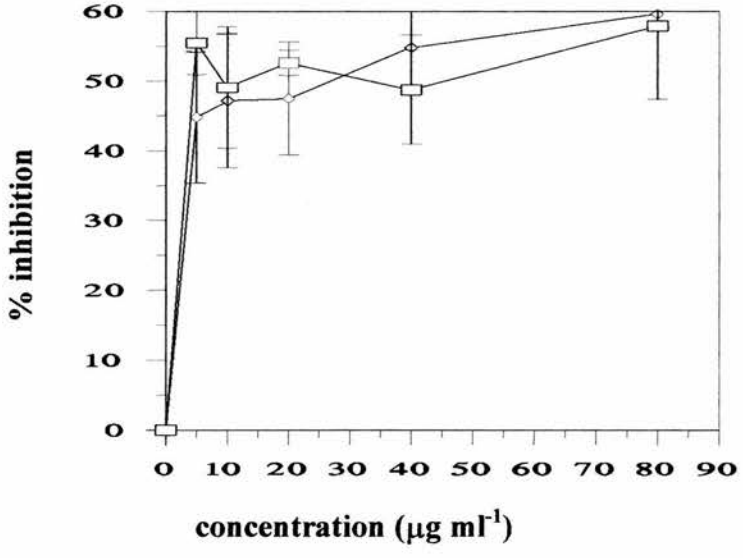
Dose response experiments with synthetic Lewis^a and Lewis^b antigens were carried out with both methods. Results of 3 experiments are shown as the mean percentage inhibition of binding in Figures 4.7a and 4.7b and the error bars represent the standard error of the mean. For *C. perfringens*, similar results for inhibition of binding by Lewis^a and Lewis^b antigens were obtained with both methods. For the *in vivo* method, significant inhibition of binding was observed for Lewis^a from 2 μg ml^{-1} 18.8% (95% CI 10.2 – 27.4, p=0.011) with a plateau of inhibition at 20 μg ml^{-1} 52.2% (95% CI 36.7 – 67.6, p=0.005). For Lewis^b, significant inhibition of binding was also observed from 2 μg ml^{-1} 15.3% (95% CI 13.8 – 16.8, p=0.001) with a plateau of inhibition at 20 μg ml^{-1} 51.9% (95% CI 32.4 – 71.4, p=0.008). For the *in vitro* method, significant inhibition of binding was observed for Lewis^a from 4 μg ml^{-1} 40.0% (95% CI 29.0 – 51.0, p=0.004) with a plateau at 10 μg ml^{-1} 47.2% (95% CI 5.8 – 88.5, p=0.039). For Lewis^b, significant inhibition of binding was observed from 2 μg ml^{-1} 24.1% (95% CI 8.0 – 40.2, p=0.023) with a plateau at 5 μg ml^{-1} 55.5% (95% CI 35.8 – 75.2, p=0.007).

Figure 4.7 Inhibition of binding of *C. perfringens* by the synthetic Lewis[a] (diamond) and Lewis[b] (rectangle) antigens (0 - 80 μg ml^{-1}): a) *in vivo* method b) *in vitro* method

a)



b)



4.3.11 Addition of synthetic Lewis^a or Lewis^b antigens to infant formula

Synthetic Lewis^a or Lewis^b antigens were added to two different dilutions of infant formula (1 in 10 and 1 in 100) to give a final concentration of 20 μg ml^{-1}. This concentration was chosen since maximum inhibition of binding was observed with this concentration for the *in vivo* methods using the synthetic Lewis antigens (4.3.10). These preparations were used in the *in vivo* system to investigate the potential for improving the inhibitory activity of infant formula against *C. perfringens* binding to epithelial cells. Positive controls included in these experiments consisted of cells, bacteria and PBS; cells, bacteria and Lewis antigen; cells, bacteria and the appropriate dilution of formula. The test samples contained cells, bacteria, the appropriate dilution of formula and Lewis^a or Lewis^b antigens.

The mean percentage inhibition of binding for 4 experiments are shown in Table 4.6. Although the addition of Lewis^a or Lewis^b to infant formula increased the observed inhibition of binding compared to infant formula alone, there was no significant difference in binding between infant formula alone and infant formula plus synthetic Lewis^a or Lewis^b.

Table 4.6 Inhibition of bacterial binding (*in vivo* method) by infant formula and infant formula containing 20 μg ml^{-1} of synthetic Lewis[a] or Lewis[b]. The p value represents the difference between equivalent dilutions of infant formula and infant formula with Lewis antigen added (mean of 4 experiments).

Condition	Mean % inhibition of binding
Formula diluted 1 in 10	16.7
Formula diluted 1 in 10 plus Lewis[a]	23.7 ($p = 0.119$)
Formula diluted 1 in 10 plus Lewis[b]	25.6 ($p = 0.061$)
Formula diluted 1 in 100	12.9
Formula diluted 1 in 100 plus Lewis[a]	24.1 ($p = 0.053$)
Formula diluted 1 in 100 plus Lewis[b]	24.2 ($p = 0.200$)

4.4 Discussion

For *C. perfringens*, both methods gave similar results for whole and defatted human milk. Enhancement of binding rather than inhibition of binding was observed and flow cytometry studies suggest that enhanced binding occurs due to the formation of bacterial aggregates. Greater enhancement of binding was observed using the *in vitro* method because bacterial aggregates are formed when they are incubated with milk or formula. Significantly different results were obtained with infant formula. The *in vivo* method showed inhibition of binding at the lower dilutions regardless of formula type. The *in vitro* method showed enhancement of binding. The results with *C. perfringens* are similar to those observed with *S. aureus*.

Oligosaccharides and glycoconjugates present in human milk have been found to inhibit binding of certain enteric pathogens and their toxins by acting as receptor analogues [Goldman, 1993]. Pre-incubation of epithelial cells with fucose containing glycopeptides from lactoferrin was found to inhibit adherence of *Shigella flexneri* to intestinal cells [Izhar *et al.*, 1982]. This suggests fucose containing components are binding to a receptor on the cell and blocking adherence. Fucosyl containing oligosaccharides protected against the heat-stable enterotoxin of *E. coli* [Cleary *et al.*, 1983]. Breast feeding has also been found to be protective against salmonella. Human milk antibodies to *Clostridium difficile* have been found to inhibit the binding of toxin A to its intestinal receptor. Bacteria producing soluble exotoxins have been found in the gastrointestinal tract of some SIDS infants

[Bettelheim *et al.*, 1989; Bettelheim *et al*, 1990; Bettiol *et al.*, 1994; Lindsay *et al*, 1994; Rolfe *et al.*, 1995].

Inhibition of binding of *C. perfringens* to cells pre-treated with anti-Lewis^a (14.7%) or anti-Lewis^b (20.5%) was not as marked as that observed for *S. aureus* (49.7% for anti-Lewis^a and 52.1% for anti-Lewis^b).

In this study, a dose response effect was observed for inhibition of binding of *C. perfringens* by the synthetic Lewis^a and Lewis^b antigens. These antigens are components of breast milk and infant formula. Higher levels of the Lewis antigens are required to cause maximum inhibition of binding using the *in vivo* protocol in which the bacteria can bind either to the Lewis antigen on the cell surface or to antigens in solution. In the *in vitro* protocol, direct blocking of the bacterial adhesin occurs before addition to the cells.

Density of colonisation is an important factor in development of bacterial disease due to invasion or toxin production [Beachey, 1981]. This study suggests that the Lewis antigens in both human milk and infant formula might reduce binding of *C. perfringens* to epithelial cells. The results obtained for synthetic Lewis antigens with the two methods follow the same trends as those observed for *S. aureus* in the previous chapter.

Breast milk contains much higher levels of the Lewis antigens compared to infant formula or cows' milk [Chapter Three]. Based on these results and the inhibition of binding studies with the Lewis antigens, the following hypothesis was tested: the inhibitory activity of infant formula in the *in vivo* system might be increased by the addition of Lewis antigens to the formula preparation. Although greater inhibition of bacterial binding was observed by the addition of Lewis antigens to the formula, no significant differences in binding were observed between formula alone and formula plus the synthetic Lewis antigens. It might be that higher concentrations of the Lewis antigens need to be added before significant differences in binding are observed. The cost of the antigens limited the numbers of experiments and concentrations tested.

Significantly higher levels of *C. perfringens* and their toxins have been identified in SIDS cases compared to non-SIDS cases [Lindsay *et al.*, 1993; Murrell *et al.*, 1993] and formula fed infants had a significantly higher incidence of *C. perfringens* and its enterotoxin in their faeces than breast fed infants [Murrell *et al.*, 1993]. Compared with healthy infants, enterotoxigenic strains of *S. aureus* and their toxins have also been isolated significantly more often from the gastrointestinal tracts of SIDS infants. Formula fed SIDS infants had a higher incidence of *S. aureus* and its enterotoxin than breast fed babies, but the increase was not significant [Murrell *et al.*, 1993].

In conclusion, although the free oligosaccharides Lewis^a and Lewis^b were shown to inhibit binding of *C. perfringens*, the major protective effect of whole human milk is likely to be due to aggregation of the bacteria by antibodies and glycoconjugates. This could result in enhanced clearance by phagocytic cells or other innate mechanisms such as peristalsis in the gut. Addition of these antigens to formula preparations might enhance the inhibition noted in the *in vivo* method; however, the concentration of Lewis antigens which showed maximum inhibition alone had no significant effect when mixed with the formula preparation. Higher concentrations of the antigen need to be tested, but the cost of the synthetic antigens did not permit further studies. These studies should be extended to other enterotoxigenic bacteria implicated in SIDS.

Chapter Five

Detection of IgA antibodies in human milk that bind to bacterial toxins implicated in SIDS

5.1 Introduction

Bacteria that produce soluble toxins on respiratory or gastrointestinal mucosa have been implicated in a significant number of cases of SIDS [reviewed by Blackwell *et al.*, 1994; 1995a, b]. Toxins of *S. aureus* and *C. perfringens* have been identified in tissues and body fluids of some SIDS victims [Murrell *et al.*, 1987; Malam *et al.*, 1992; Lindsay *et al.*, 1993; Zorgani *et al.*, 1999]. These toxins can act as superantigens and have been suggested to play a role in precipitating the events leading to some SIDS deaths [Lindsay, 1996]. *C. perfringens* does not invade healthy cells but produces toxins and *C. perfringens* enterotoxin interacts with the cell membrane causing pore formation [Petit *et al.*, 1999].

Breast fed infants are at a lower risk of dying from SIDS compared to formula fed infants [Ford *et al.*, 1993] and breast feeding has been suggested to enhance secretory immunity of mucosal surfaces during the early neonatal period [Stephens, 1986] as development of the secretory IgA system does not occur until some time after birth [Goldman, 1993].

Secretory IgA is the predominant immunoglobulin in breast milk [Goldman, 1993] and plays an important role as a specific defence mechanism for the breast fed baby by providing local immunological protection at mucosal surfaces against bacterial or viral infection or by preventing antigens crossing the epithelial barrier [Jatsyk *et al.*, 1985; Vassilev *et al.*, 1996]. Levels of secretory IgA are highest in colostrum, and concentrations of secretory IgA in milk decrease with time as an infant's own

immune system begins to provide protection against potential pathogens [Davidson *et al.*, 1987].

The agglutination of *S. aureus* and *C. perfringens* by human milk was observed in 3.3.11 and 4.3.9. Another protective effect of human milk could be the neutralising activities of secretory antibodies for toxins which are not present in infant formula preparations. Antibodies to the heat stable enterotoxin of *Escherichia coli* have been identified in human milk and these antibodies protected suckling mice from the lethal effects of the toxin [Cleary *et al.*, 1983]. Both non-immunoglobulin and immunoglobulin components of human milk were found to inhibit binding of *Clostridium difficile* toxin A to its receptor [Rolfe *et al.*, 1995].

The aim of this work was to examine individual samples of human milk and different infant formula preparations to quantitate levels of IgA specific for the staphylococcal toxins toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin C (SEC) and *C. perfringens* enterotoxin A (CEA). Antibody levels against TSST-1 and SEC were compared with the isolation of *S. aureus* from the mother's nose or throat.

In non-ruminant species, the main immunoglobulin present in external secretions and involved in mucosal immunity is IgA. In cattle and sheep however, IgG_1 is the predominant immunoglobulin in external secretions and is more important than IgA in the mucosal immune system of ruminants. This is especially evident in the

ruminant mammary gland which has the least well developed IgA system of all the secretory organs. Shortly before parturition, there is selective transfer of IgG_1 from blood serum and IgG_1 is present in high levels in both the ruminant mammary gland and colostrum. IgG_1 is crucial for protection of the newborn against various pathogens and maternally derived IgG_1 protects young animals against colibacillosis and rotavirus infections [Lascelles *et al.*, 1986]. Infant formula preparations are made from skimmed cows' milk or skimmed cows' milk powder and were not expected to contain human IgA antibodies; however, infant formula preparations might contain bovine IgG_1. This was assessed in both infant formula and cows' milk. Samples of human milk were included as a negative control since these should not contain any bovine antibodies.

5.2 Materials and methods

5.2.1 ELISA for detection of specific human IgA against staphylococcal and clostridial toxins in human milk and infant formula

The wells of microtitre plates (Greiner) were coated with 50 µl of the individual toxin (1 µg ml^{-1}) in coating buffer, TSST-1, SEC or CEA. Controls included wells coated with 50 µl of serial dilutions of a standard human IgA derived from colostrum (Sigma); concentrations ranged from 9 – 9,200 ng ml^{-1}. The plates were incubated overnight at 4°C.

The plates were washed with washing buffer and blocked with 50 µl of blocking buffer for 30 min at room temperature. The plates were washed and 50 µl of undiluted human milk or infant formula preparations or dilutions of 1 in 5, 1 in 10 or 1 in 100 in blocking buffer were added to the appropriate wells. Blocking buffer (50 µl) was added to the standard and TSST-1 and SEC control wells. For the CEA plates, 50 µl of *C. perfringens* anti-toxin (kindly supplied by Mr R. Brown of this Department) developed in horse and diluted 1 in 100 in blocking buffer was added. All plates were incubated for 2 h at room temperature.

After washing, 50 µl of HRP-labelled goat anti-human IgA (Sigma) diluted 1 in 100 in blocking buffer were added to the test and standard wells. To the control wells which contained toxin but no milk or infant formula sample, for TSST-1 and SEC, 50 µl of HRP-labelled sheep anti-TSST-1 (Toxin Technology) (diluted 1 in 300 in

blocking buffer) or HRP-labelled sheep anti-SEC (Toxin Technology) (diluted 1 in 600 in blocking buffer) were added respectively. For CEA, 50 µl of HRP-labelled rabbit anti-horse IgG (diluted 1 in 100 in blocking buffer) (Sigma) were added. All plates were incubated for 2 h at RT. The plates were washed and the experiment continued as described in 2.6. The samples were tested in duplicate. The absorbency at 490 nm (A_{490}) was measured on an ELISA plate reader (Dynatech), the results analysed and the immunoglobulin concentrations (ng ml^{-1}) were determined by comparing their absorbency with the linear part of the standard curve run for each experiment.

5.2.2 ELISA for detection of specific bovine IgG_1 against staphylococcal and clostridial toxins in cows' milk, infant formula and human milk

Plates were coated with the appropriate toxin as described in 5.2.1. Controls included wells coated with 50 µl of various concentrations of bovine immunoglobulin reference serum (Bethyl Laboratories, Universal Biologicals Limited) ranging for IgG_1 from 2.3 - 45,200 ng ml^{-1}. The plates were incubated overnight at 4°C.

The plates were washed with washing buffer and blocked with 50 µl of blocking buffer for 30 min at room temperature. The plates were washed and 50 µl of undiluted cows' milk, infant formula preparations or human milk were added to the wells. Dilutions of these (1 in 10; 1 in 100; 1 in 1,000 in blocking buffer) were also

added to the appropriate wells. The positive control wells were treated as described in 5.2.1. All plates were incubated for 2 h at room temperature.

After washing, 50 µl of HRP-labelled sheep anti-bovine IgG_1 (Bethyl Laboratories, Universal Biologicals Limited) diluted 1 in 100 in blocking buffer was added to the test and standard wells. The control wells were treated as described in 5.2.1. All plates were incubated for 2 h at room temperature.

The plates were washed and the experiment continued as described in 2.6. The samples were tested in duplicate. The absorbency at 490 nm (A_{490}) was measured on an ELISA plate reader (Dynatech), the results analysed and the IgG_1 immunoglobulin concentrations (ng ml^{-1}) were determined by comparing their absorbency with the linear part of the standard curve run for each experiment.

5.3 Results

5.3.1 Detection of human IgA bound to TSST-1, SEC and CEA in human milk and infant formula by ELISA

Individual breast milk samples were tested for the presence of IgA antibodies that bind to TSST-1 (n = 33), SEC (n = 32) and CEA (n = 28). Preliminary work with TSST-1 used whole and defatted pools of human milk (Figure 5.1). The whole milk pool gave higher results than the defatted pool; therefore, whole milk samples were used. Individual milk samples were tested without dilution and at dilutions of 1 in 5, 1 in 10 and 1 in 100 to try to dilute out the inhibitory activity of the milk observed at lower dilutions (Figure 5.1).

The results obtained for IgA binding to TSST-1 are shown in Figure 5.2. The levels of IgA binding to TSST-1 ranged from 900 – 3,100 ng ml^{-1} with a mean of 1,788 ng ml^{-1} (SD ± 587). The results obtained for IgA binding to SEC are shown in Figure 5.3. The levels of IgA binding to SEC ranged from 1,000 – 3,600 ng ml^{-1} with a mean of 2,009 ng ml^{-1} (SD ± 618). The results obtained for IgA binding to CEA are shown in Figure 5.4. The levels of IgA binding to CEA ranged from 1,000 – 4,300 ng ml^{-1} with a mean of 1,718 ng ml^{-1} (SD ± 636).

Figure 5.1 IgA antibodies bound to TSST-1 in pooled whole and defatted human milk (dilution 1 = neat; dilution 2 = 1 in 5; dilution 3 = 1 in 10; dilution 4 = 1 in 100; dilution 5 = 1 in 1,000)

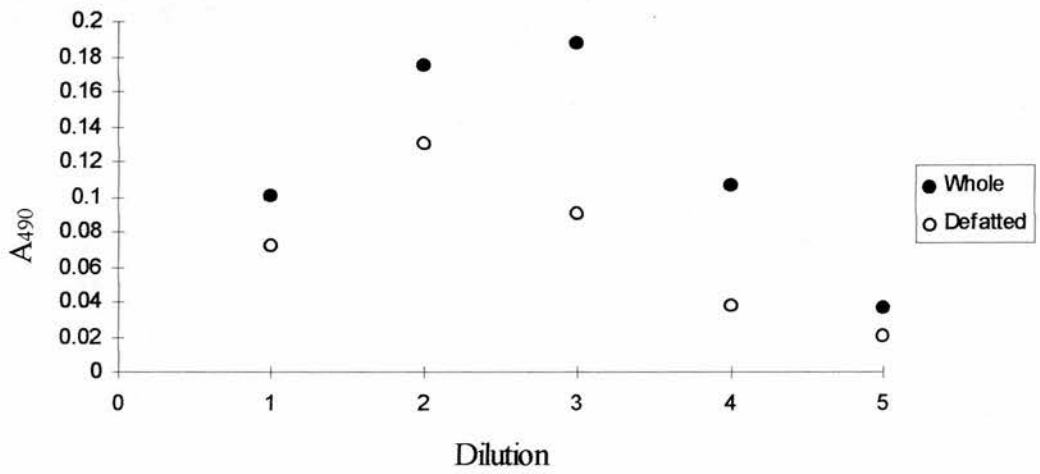


Figure 5.2 IgA antibodies bound to TSST-1 in individual samples of breast milk

(–) = no *S. aureus* isolated, (♦) = *S. aureus* isolated

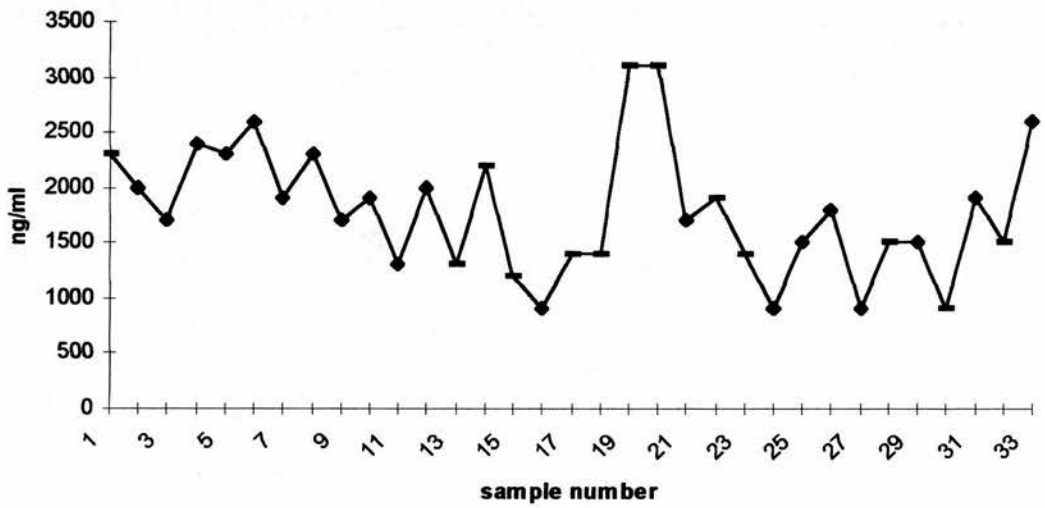


Figure 5.3 IgA antibodies bound to SEC in individual samples of breast milk

(–) = no *S. aureus* isolated, (♦) = *S. aureus* isolated

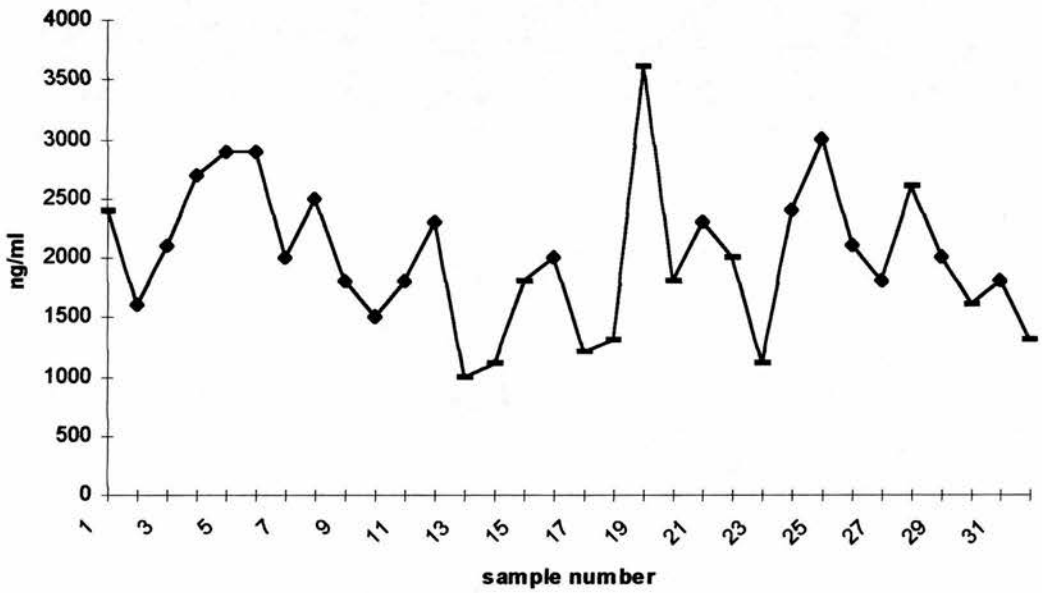
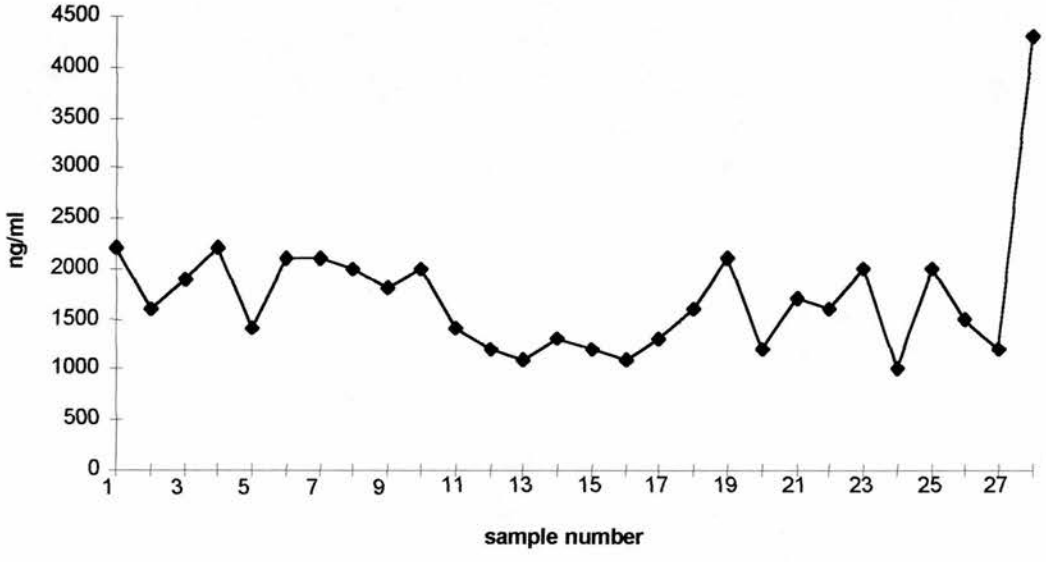


Figure 5.4 IgA antibodies bound to CEA in individual samples of breast milk



There were 28 individual samples with sufficient milk to test for IgA antibodies against the three toxins. Of these 28 samples, the levels of IgA antibodies that bound to TSST-1 were highest in five (17.8%) compared with either SEC or CEA. In one sample (20%), the levels of IgA binding to TSST-1 were highest followed by SEC, then CEA. In three samples (60%), the levels of IgA binding to TSST-1 were highest followed by CEA, then SEC. For the remaining sample (20%), the levels of IgA binding to TSST-1 were highest followed by equal levels of SEC and CEA.

Of the 28 samples, 17 (60.7%) contained higher levels of IgA that bound to SEC compared with either TSST-1 or CEA. In nine of the 17 samples (52.9%), the levels of IgA binding to SEC were highest followed by TSST-1, then CEA. In six of the 17 samples (35.3%), the antibody levels against SEC were highest followed by CEA, then TSST-1 and in two of the 17 samples (11.8%), the levels of IgA binding to SEC were highest followed by equal levels of TSST-1 and CEA.

There were five of the 28 samples (17.8%) which contained highest levels of IgA antibodies to CEA compared with either TSST-1 or SEC. In three samples (60%), the levels of IgA bound to CEA were highest followed by TSST-1, then SEC. In two samples (40%), the highest levels of IgA were bound to CEA followed by SEC, then TSST-1.

One of the 28 samples had equal levels of IgA bound to SEC and CEA but lower levels of IgA bound to TSST-1.

Five different infant formula preparations were examined as negative controls for the presence of human IgA against TSST-1, SEC and CEA. No specific IgA was detected in any of the infant formula preparations tested. This was the expected result since infant formulas are prepared from skimmed cows' milk or skimmed cows' milk powder.

5.3.2 Isolation of S. aureus from the mother and detection of human IgA to staphylococcal toxins

The breast milk samples were collected at either visit one or two during the first 6 months after the infant was born. The IgA levels bound to TSST-1 and SEC were assessed with reference to the presence or absence of *S. aureus* in the mother's nose or throat. Samples 24 and 25, and samples 26 and 27 were taken from the same mother at different times due to their becoming involved in the study for the second time after the birth of another child.

For the 33 samples tested for IgA binding to TSST-1, 17 (51.5%) had concentrations less than the mean of 1,788 ng ml^{-1} (SD ± 587). Of these, eight (47.1%) had no *S. aureus* isolated at either visit and nine (52.9%) had *S. aureus* isolated at one or more visits. Of 16 samples with concentrations greater than or equal to 1,788 ng ml^{-1}, five (31.2%) had no *S. aureus* isolated at either visit and 11 (68.8%) had *S. aureus* isolated at one or more visits. There were five of the 33 samples (15.1%) (numbers 4, 6, 19, 20 and 33) that contained IgA levels greater than the upper limits of the

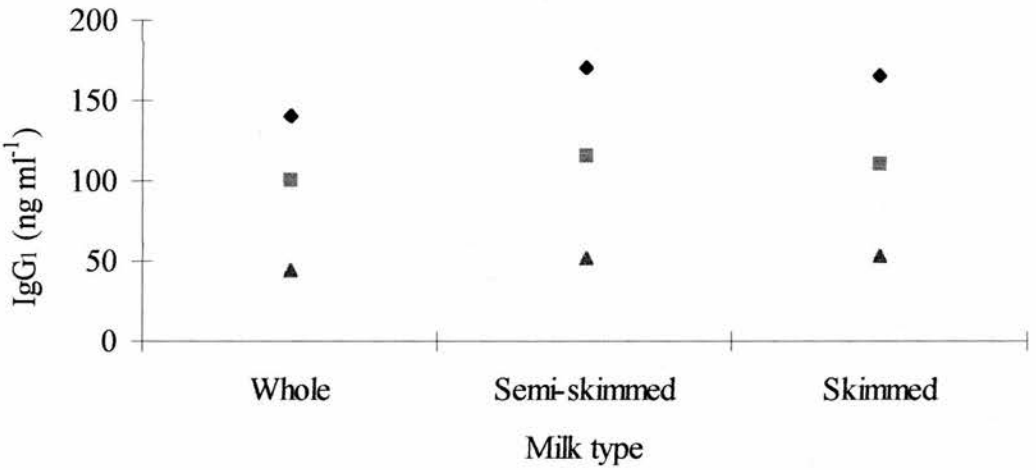
standard deviation (2,375 ng ml^{-1}) of IgA bound to TSST-1. *S. aureus* was not isolated from two of the mothers (40%) (numbers 19 and 20) at either visit; however, for the remaining three samples (60%) (numbers 4, 6 and 33), *S. aureus* was isolated from the mother at one or more visits. There were five samples (15.1%) (numbers 15, 16, 24, 27 and 30) that had IgA levels less than the lower limits of the standard deviation (1,201 ng ml^{-1}). Of these samples, two (40%) of the mothers (numbers 15 and 30) had no *S. aureus* isolated at either visit whilst the other three samples (60%) came from mothers who had *S. aureus* isolated at one or more visits.

For 32 samples tested for IgA bound to SEC, 19 (59.4%) had concentrations less than the mean of 2,009 ng ml^{-1} (SD ± 618). Of these, 10 donors (52.6%) had no *S. aureus* isolated at either visit but the remaining nine (47.4%) had *S. aureus* isolated from the mother at one or more visits. There were 13 samples (40.6%) with IgA levels greater than or equal to 2,009 ng ml^{-1}. Of these, three donors (23.1%) had no *S. aureus* isolated at either visit and ten of the mothers (76.9%) had *S. aureus* isolated at one or more visits. There were five of the 32 samples (15.6%) (numbers 4, 5, 6, 19 and 25) that had IgA levels above the upper limits of the standard deviation (2,627 ng ml^{-1}). For one sample (number 19), no *S. aureus* was isolated from the mother at either visit. The other 4 samples came from mothers who had *S. aureus* isolated at one or more visits. There were five samples (15.6%) that had IgA levels below the lower limits of the standard deviation (1,391 ng ml^{-1}) and all 5 donors were culture negative for *S. aureus* at both visits (Fisher's exact test $p < 0.05$).

5.3.3 Detection of bovine IgG_1 bound to TSST-1, SEC and CEA in cows' milk, infant formula and human milk by ELISA

Bovine IgG_1 antibodies against TSST-1, SEC or CEA were not detected in any of the different infant formula preparations (n = 5) or human milk samples (n = 4) tested. Bovine IgG_1 antibodies which bound to TSST-1, SEC and CEA, were detected in whole, semi-skimmed and skimmed cows' milk. Highest levels were bound to TSST-1, followed by SEC and then CEA. The results for the different cows' milk samples are shown in Figure 5.5.

Figure 5.5 IgG_1 antibodies bound to TSST-1, SEC and CEA in whole, semi-skimmed and skimmed milk (♦ = TSST-1, ■ = SEC, ▲ = CEA) (for the 1 in 10 dilution)



5.4 Discussion

Secretory IgA has been suggested to act locally in the infant gut as a first line of defence against foreign antigens. This might occur by binding to gastrointestinal pathogens and interfering with their attachment to mucosal cells [Davidson *et al.*, 1987]. Secretory IgA is well adapted to persist in the gastrointestinal tract since it is resistant to digestive enzymes [Goldman, 1993] as shown by its detection in faeces [Davidson *et al.*, 1987]. Secretory IgA is resistant to digestion because of its stable molecular arrangement [Lönnerdal, 1996]. No immunoglobulins have been found in the intestinal tracts of bottle fed infants until they produce their own secretory IgA [Jatsyk *et al.*, 1985]. Breast fed infants have been found to produce their own intestinal secretory IgA sooner than formula fed infants [Dallas *et al.*, 1998].

Specific IgA antibodies in breast milk have been found against common microorganisms present in the gastrointestinal or respiratory tracts [Goldman, 1993]. A number of studies have found that breast feeding protects infants against pathogenic species including *Campylobacter*, *Shigella*, *E. coli*, respiratory syncitial virus (RSV), *Haemophilus influenzae* and *Helicobacter pylori* [van Rosen *et al.*, 1990, Thomas *et al.*, 1993; Buescher 1994]. IgA is thought to be responsible for this protection, and an association between the absence of specific immunoglobulin in human milk and the occurrence of symptomatic infections caused by these bacteria and viruses has been observed [Buescher, 1994]. Evidence has been obtained which found that mothers' produce "environmentally specific" milk for their infants. Levels of secretory IgA in breast milk are increased upon maternal exposure to

specific pathogens and means that infants receive antibodies directed against specific pathogens. These antibodies provide an infant with protection during a time when their immune system is not fully developed [Lawrence, 1994].

Studies have also found that by binding to bacterial toxins, secretory IgA can neutralise their activity [Kim *et al.*, 1984; Rolfe *et al.*, 1995; Dallas *et al.*, 1998]. Secretory IgA has been found to neutralise the effects of *Clostridium difficile* toxin A [Kim *et al.*, 1984] and this was suggested to occur through inhibition or modification of binding of toxin A to its intestinal receptor [Rolfe *et al.*, 1995]. Later work by the same group found that toxin A binds to the secretory component which acts as a receptor analogue [Dallas *et al.*, 1998]. Human milk has also been found to prevent *Vibrio cholerae* toxin and heat-stable *E. coli* enterotoxin causing pathogenic effects without preventing colonisation by the bacteria in question [Dallas *et al.*, 1998].

The levels of IgA detected in individual breast milk samples varied among mothers, and these different levels of protective antibodies might confer different degrees of protection to an infant against bacterial toxins. In most samples the highest levels of IgA were detected in the ELISA with SEC; however, the highest IgA level detected was 4,300 ng ml^{-1} and was against CEA. It was not possible to test the milk samples for their ability to neutralise the toxins in a model system using human buffy coats because both whole and defatted milk elicited production of strong inflammatory responses from the cells in the absence of toxin; however, the $Lewis^a$ and $Lewis^b$

antigens which were found in the milk samples were examined for their effect on TSST-1 [Chapter Six].

While *S. aureus* was isolated from about two thirds of women whose milk samples had levels of IgA to the toxin greater than or equal to the mean, the difference was not significant compared to isolation rates from women whose milk samples had levels below the mean. The only exception was found for SEC. Five samples had IgA levels above the upper limits of the standard deviation and of these, *S. aureus* was isolated from 4 of the mothers. Five samples were also found to have IgA levels below the lower limits of the standard deviation and all five were culture negative for *S. aureus*.

Some samples were found to have low levels of specific antibody to TSST-1 and / or SEC and had been taken when *S. aureus* was isolated from the mother (Figures 5.2 and 5.3). Two explanations can be suggested: 1) *S. aureus* isolated from these mothers did not produce the relevant toxins; 2) the mothers were recently colonised and had not yet developed IgA to the toxins.

For 20 women in the 20-29 and 30-39 age ranges, the mean levels of serum IgG bound to TSST-1 were 5,150 ng ml^{-1} and 4,050 ng ml^{-1} respectively and for SEC, 4,200 ng ml^{-1} and 2,850 ng ml^{-1} respectively [Al Madani, 1999]. This indicated that the majority of women of child-bearing age have encountered the toxins and have produced a good humoral response. Levels of secretory IgA produced in response to

an antigen decrease with time if the stimulus is no longer present. The higher proportion of carriers among women with IgA levels to TSST-1 (68.8%) or SEC (76.9%) greater than or equal to the mean indicate that carriage of *S. aureus* by the mother might have a booster effect on secretory IgA responses. Greater numbers of women need to be assessed to obtain evidence for this hypothesis.

In comparison to non-ruminant species, cows' milk contains very little IgA [Wharton *et al.*, 1994]. IgG_1 is the main immunoglobulin involved in the mucosal immune system of ruminants and is of particular importance in protecting young ruminant animals against various pathogens [Lascelles *et al.*, 1986]. Pregnant cows have been immunised with a polyvalent *E. coli* vaccine in an attempt to increase the immunoglobulin content of cows' milk; however, although an extra antibody was produced, it was IgG and not secretory IgA [Wharton *et al.*, 1994]. IgG_1 was detected in cows' milk but not in any of the infant formula preparations or human milk samples tested. Thus, formula fed infants will not have any protective antibodies, either human or bovine, in their intestinal tracts against infectious agents or their products, until they produce their own secretory IgA.

In conclusion, if bacterial toxins play a role in precipitating a SIDS death, the presence in breast milk of IgA antibodies which could neutralise bacterial toxins implicated in SIDS might contribute to the reported protective effect of breast feeding in relation to these infant deaths.

Chapter Six

The prevalence of night-time deaths in SIDS: Development of a system to assess factors that could control inflammatory responses to toxic shock syndrome toxin-1 (TSST-1)

6.1 Introduction

It has been proposed that some SIDS deaths are due to uncontrolled inflammatory reactions in response to infectious agents and / or cigarette smoke [Blackwell *et al.*, 1995a]. Major candidates for triggering these reactions are pyrogenic toxins of *S. aureus*. These toxins have been identified in tissues of over half the SIDS infants tested and from cases of unexpected deaths in adults and older children [Newbould *et al.*, 1989; Malam *et al.*, 1992; Bentley *et al.*, 1997; Zorgani *et al.*, 1999]. They have superantigenic activities that can induce shock and death in previously healthy adults [Schlievert, 1995].

There is evidence for mild infection and associated inflammatory responses in SIDS infants. As a group, SIDS victims had evidence of increased inflammatory stimulation in the upper airways and intestinal tract compared with infants who died of other causes such as violent deaths. Pro-inflammatory cytokines, interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), have been detected in body fluids or tissues of SIDS victims. At autopsy, there are often signs of mild inflammatory reactions and increased levels of some acute phase proteins, increased production of immunoglobulins in the respiratory tract and gut, and changes indicating T-cell activation [Forsyth *et al.*, 1999; Vege *et al.*, 1999].

The majority of SIDS deaths occur during the early hours of the morning and it has been suggested that sleep might be involved in the mechanism of some SIDS deaths [Schechtman *et al.*, 1995; Cornwell *et al.*, 1998]. Studies on the physiology of sleep

in infants have produced important clues to relate developmental stage with the proposed role of inflammation in SIDS.

The 2 – 4 month age range has been consistently identified as the period in which the majority of SIDS deaths occur. During this age range, infants develop adult-like night-time temperature patterns and diurnal variation in hormone levels. In normal infants, between 7 and 16 weeks, the body temperature falls at night to 36.4°C, similar to that of sleeping adults; however, this change can occur as late as 22 weeks among infants of low birthweight or who experienced problems at delivery [Lodemore *et al.*, 1992]. Infants who remain in the "immature" state share many risk factors with SIDS infants, and Wailoo and colleagues have suggested that the "immature state" is a risk factor for SIDS. In contrast, other work by this group found that Asian infants stay in the "immature" stage significantly longer than white infants [Petersen *et al.*, 1994], but Asian infants have been found to have a lower incidence of SIDS [Balarajan *et al.*, 1989].

The findings of Wailoo and colleagues can also be explained by relating them to other physiological changes that occur in conjunction with the change in body temperature rhythm. The week following the switch to an adult-like night-time temperature pattern, there is a dramatic drop in night-time, but not daytime, cortisol levels. Before the developmental change, the morning and night-time values for cortisol are 0.34 – 22.5 $\mu g\ dl^{-1}$ and 0.22 – 15.2 $\mu g\ dl^{-1}$ respectively. After the developmental switch, there is no significant change in the daytime levels

0.34 – 23.9 μg dl^{-1} but a sharp drop in the night-time levels to 0.1 – 7.25 μg dl^{-1} is observed.

Cortisol and other glucocorticoids play a key role in suppression of almost all components of the inflammatory responses. In infants, the adrenal gland decreases in weight during the first two months after birth. This is associated with a decrease in plasma cortisol levels [Wittekind *et al.*, 1993].

Hormone levels associated with circadian rhythm affect inflammatory responses in adults. Adults are less able to control inflammatory responses such as TNF responses to endotoxin during the early hours of the morning when there is minimal secretion of cortisol since the gland is less responsive after midnight [Pollmacher *et al.*, 1996]. It is during these hours that a significant number of SIDS deaths occur. Inflammatory responses induced by endotoxin from blood samples of individuals with obstructive sleep apnoea syndrome were compared with responses induced from samples obtained from normal individuals. TNF-α, IL-1, IL-6 and IFN-γ responses were measured in samples taken at different times during the day and night. For normal individuals, the peak of induction of these mediators occurred during late evening or early morning. For patients with obstructive sleep apnoea syndrome, the peak occurred during the afternoon or early evening. Production of high levels of inflammatory mediators corresponded to times of minimal cortisol production [Entzian *et al.*, 1996].

Breast fed infants develop adult-like night-time temperature and circadian rhythm patterns significantly earlier than formula fed infants [Lodemore *et al.*, 1992]. Breast milk contains IgA antibodies that can bind to the staphylococcal toxins TSST-1 and SEC [Chapter Five]. These antibodies might be able to reduce TSST-1's ability to induce pro-inflammatory cytokine production. Depending on secretor status, Lewis^a and Lewis^b antigens are found in high levels in breast milk, but in much lower levels in infant formula [Chapter Three]. In other studies, toxins have been demonstrated to bind to the Lewis antigens expressed on the surface of monocytes. Binding of pertussis toxin to macrophages was reduced by capping the Lewis^a antigen on cells with monoclonal antibodies to the antigen [van't Wout *et al.*, 1992]. Binding of some staphylococcal enterotoxins (TSST-1 and SEC) to monocytes can be partially inhibited by pre-treating monocytes with monoclonal anti-Lewis^a antibodies [Essery *et al.*, 1994; Essery, 1997].

The objectives of the present study were: 1) to assess in a model system the effects of breast milk, infant formula and the synthetic Lewis^a and Lewis^b antigens on induction of pro-inflammatory cytokines in response to TSST-1; 2) to investigate the effect of cortisol levels in infants found at night and during the day (both before and after development of the circadian rhythm) on inflammatory responses elicited by TSST-1.

6.2 Materials and methods

6.2.1 Source of human cells

One day old human buffy coats were obtained from the Scottish National Blood Transfusion Service (SNBTS) (Royal Infirmary of Edinburgh). The buffy coats were diluted 1 in 4 with sterile pyrogen free PBS and layered onto a Histopaque 1077 gradient (Sigma). After centrifugation at 300 x *g* for 30 min at room temperature (RT), the cells in the interface layer were removed and washed twice in Dulbeco's Modified Eagle's Medium (DMEM) at 250 x *g* for 10 min. Viable cells were counted using the trypan blue exclusion method using a Neubauer Haemocytometer and diluted to 2 x 10^6 cells ml^{-1} in DMEM plus 5% (v/v) serum from the individual donor or 5% (v/v) FCS (Gibco), 1% (w/v) L-glutamine (Gibco), penicillin (100 IU ml^{-1}) and streptomycin (200 μg ml^{-1}) (Gibco).

6.2.2 Optimisation of toxin concentration and incubation time for induction of inflammatory mediators

TSST-1 was used in all experiments. Dose response experiments were carried out to determine the optimal amount of toxin for induction of the pro-inflammatory cytokines TNF-α, TNF-β, IL-1β, IL-6 and nitric oxide. TSST-1 was tested at concentrations of 0.1 – 5 μg ml^{-1}. In these initial experiments, 50 μl of medium or TSST-1 was added to 950 μl of cells (2 x 10^6 cells ml^{-1}) resuspended in growth medium containing 5% (v/v) FCS. The samples were incubated for 0, 8, 12, 16, 24, 36, 48 and 72 h at 37°C in 5% CO_2. The tubes were centrifuged at 250 x *g* for 20

min; the supernatant fluids were stored at -20°C and the cells were stored at -70°C. The supernatants were examined for pro-inflammatory cytokine production.

6.2.3 Pasteurisation of the human milk pool and infant formula

The pasteurisation method described by Jay [1996] was adapted for human milk and infant formula. The pasteurisation process was carried out in a water bath under the following conditions of temperature and time: 63°C for 30 min; 72°C for 15 sec; 89°C for 10 sec; 100°C for 5 sec. After pasteurisation, a loopful of the human milk pool or infant formula was streaked on Columbia horse blood agar plates and incubated at 37°C for 48 h to check for the presence of any contaminating microorganisms [Wright *et al.*, 1998].

6.2.4 Preparation of the synthetic Lewis antigens

The synthetic Lewis^a and Lewis^b antigens (Dextra) were prepared by dissolving them in 1 ml of pyrogen free PBS. They were stored in 200 µl aliquots at -20°C until use. No sodium azide was added to this preparation.

6.2.5 Effect of pasteurised breast milk and infant formula on induction of inflammatory mediators

Leukocytes (1 ml), prepared as described in 6.2.1 and diluted to 2 x 10^6 cells ml^{-1} in medium containing 5% FCS, were added to 5 ml sterile culture tubes (Elkay). Negative controls containing cells with medium alone (1 ml) or cells with breast milk or infant formula alone (500 µl of the appropriate dilution of milk or formula

and 500 µl of medium) were included in each experiment. The positive control samples contained cells plus 500 µl of medium and 500 µl of toxin at a final concentration of 0.1 µg ml^{-1}. Test samples contained cells plus 500 µl of toxin and 500 µl of the appropriate milk or formula dilution. The dilutions of milk and formula used were 1 in 10 and 1 in 100.

6.2.6 Effect of synthetic Lewis antigens on induction of inflammatory mediators

6.2.6.1 Optimisation of toxin concentration and incubation time

In these initial experiments, 500 µl of medium and 500 µl of TSST-1 was added to 1 ml of cells (2 x 10^6 cells ml^{-1}) resuspended in growth medium containing 5% (v/v) serum from the individual donor. Concentrations of TSST-1 ranging from 0.1 - 1.0 µg ml^{-1} were tested and assessment of TNF-α and IL-6 production made at 0, 6 and 24 h. TSST-1 was used in all experiments involving the synthetic Lewis antigens at a final concentration of 0.5 µg ml^{-1}.

6.2.6.2 Effect of synthetic Lewis antigens

Leukocytes (1 ml) were added to 5 ml sterile culture tubes (Elkay). Negative controls containing cells with medium alone (1 ml) or Lewis antigen alone (500 µl of the appropriate Lewis antigen concentration and 500 µl of medium) were included in each experiment. The positive control samples contained cells with 500 µl of toxin and 500 µl of medium at a final concentration of 0.5 µg ml^{-1}. Test samples contained cells plus 500 µl of toxin and 500 µl of the appropriate Lewis

antigen concentration. Lewis^a and Lewis^b antigens were added to give final concentrations of 0.5, 5 and 10 μg ml^{-1}.

Results for the test samples were expressed as percentage of the positive control with toxin and cells only (100%).

6.2.7 Effect of cortisol on induction of inflammatory mediators

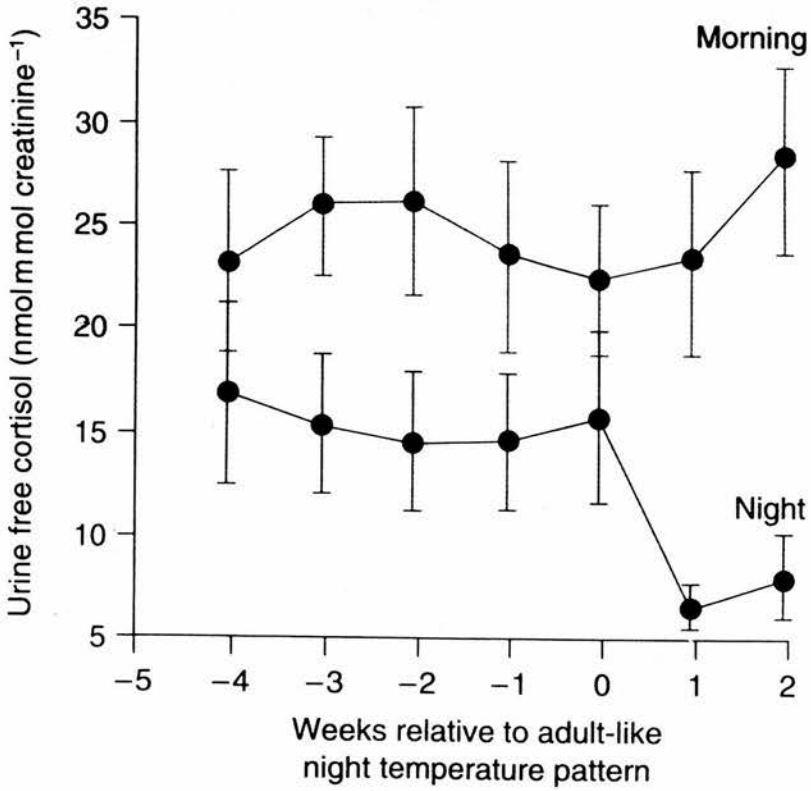
Leukocytes (1 ml) prepared in 6.2.1 were resuspended in growth medium with 5% (v/v) FCS and added to 5 ml sterile culture tubes (Elkay). Negative controls containing cells and medium alone (1 ml) or cortisol alone (500 μl cortisol and 500 μl medium) were included in each experiment. The positive control samples contained cells and 500 μl of toxin and 500 μl of medium at a final concentration of 0.1 μg ml^{-1}. Test samples contained cells and 500 μl of toxin and 500 μl of the appropriate cortisol concentration. Water-soluble hydrocortisone (Sigma) was freshly prepared in cell growth medium for each experiment to give a range of concentrations from 0.62 μg dl^{-1} to 25 μg dl^{-1}. These concentration ranges were chosen based on the graph produced by Wailoo and Petersen (Figure 6.1) [from Blackwell *et al.*, 1995a (permission from Mike Wailoo to use this graph has been requested)] which showed changes in urinary excretion of cortisol in the weeks before and after the appearance of an adult-like temperature pattern. The values were converted to represent the values of cortisol present in serum based on information from Jonetz-Mentzel *et al.*, 1993 (Table 6.1); Cole *et al.*, 1995; Sonntag *et al.*, 1996 and Orth *et al.*, 1998.

The samples were incubated for 0, 3, 6, 9, 12, 24, 48 and 72 h and the supernatants and cells collected and stored as described in 6.2.3. The supernatants were tested for pro-inflammatory cytokine production and the results for the test samples treated with cortisol expressed as a percentage of the control containing toxin alone (100%).

Table 6.1 Reference ranges for cortisol in serum of infants [Jonetz-Mentzel *et al.*, 1993]

Age	Cortisol ($\mu g\ dl^{-1}$)
5 days	0.55 – 19.8
2 – 12 months	2.38 – 22.9

Figure 6.1 Urinary free cortisol excretion in relation to circadian rhythm pattern development [Blackwell *et al.*, 1995a]



6.2.8 ELISAs for detection of inflammatory mediators

6.2.8.1 Detection of IL-6 production by ELISA

The wells of microtitre plates (Greiner) were coated with 50 µl of mouse monoclonal anti-IL-6 (R&D Systems, UK) diluted 1 in 500 to 1 µg ml^{-1} in coating buffer. After incubation overnight at 4°C, the wells were washed 3 times with washing buffer. The plates were blocked with 50 µl of blocking buffer at room temperature for 30 min. The wells were washed 3 times and 50 µl of the test samples added to duplicate wells. For each plate, dilutions of standard recombinant human IL-6 (R&D) ranging from 0.19 – 100 ng ml^{-1} were made in DMEM medium, added to duplicate wells and incubated for 2 h with continuous shaking in an orbital incubator at 37°C. The plates were washed 3 times and 50 µl of goat polyclonal anti-IL-6 antibody (R&D) diluted 1 in 1,000 to 1 µg ml^{-1} in blocking buffer was added to each well. The plates were incubated at 37°C for 2 h with continuous shaking, washed 3 times and 50 µl of HRP-conjugated donkey anti-sheep/goat IgG (SAPU) diluted 1 in 100 in blocking buffer was added to each well. The plates were incubated at 37°C for 1 h, washed 3 times and the plates were developed as described in 2.5.6. The absorbency at 490 nm was measured using an ELISA plate reader (Dynatech). The concentration of IL-6 (ng ml^{-1}) in each sample was obtained by comparing the absorbency obtained with those of the standard curve obtained with the recombinant IL-6. The results of duplicate samples were averaged and the results expressed in ng ml^{-1}.

6.2.8.2 Detection of IL-1β and TNF-β production by ELISA

The ELISA protocol for detection of IL-6 production was used to test for production of IL-1β and TNF-β and the appropriate monoclonal and polyclonal antibodies and recombinant standards were used as described in Table 2.1.

6.2.9. Bioassay for detection of TNF-α production

This method was adapted from the technique of Delahooke *et al*; [1995].

6.2.9.1 L929 cells

The mouse fibroblast cell line L929 obtained from the European Collection of Animal Cell Cultures was used for TNF-α bioassays. Cells were grown in 75 cm^3 tissue culture flasks (Greiner) at 37°C with 5% CO_2 in growth medium (GM) containing DMEM (Sigma) containing 5% (v/v) FCS (Gibco), 1% (w/v) L-glutamine (Gibco), penicillin (100 IU ml^{-1}) and streptomycin (200 μg ml^{-1}) (Gibco). Cells were split once semi-confluent growth was reached.

6.2.9.2 Day one

Growth medium was discarded and the monolayer washed twice with sterile PBS. To disperse the cells 0.005% (w/v) trypsin - 0.02% (w/v) EDTA (1.5 ml) was added for 30 sec, discarded and the cells left for 5 - 10 min to detach. The cells were resuspended in growth medium and washed twice to remove trypsin. The cells were counted at a 1 in 10 dilution in trypan blue with a Neubauer counting chamber and the cell count adjusted to 3 x 10^5 cells ml^{-1} in the growth medium. Cells (100 μl)

were added to 96-well tissue culture plates; 6 wells per plate contained medium only to act as negative controls. The plates were incubated at 37°C, 5% CO_2 for 24 h.

6.2.9.3 Day two

The medium was aspirated from all wells and replaced with 100 µl of assay medium containing RPMI 1640 (Gibco) supplemented with 5% (v/v) FCS (Gibco) and 1% (w/v) L-glutamine (Gibco) and actinomycin D (2 µg ml^{-1}) (Sigma). The plates were incubated for 2 h at 37°C with 5% CO_2. The medium was aspirated and 100 µl of test supernatant (diluted 1 in 2 or 1 in 4) in assay medium without actinomycin D added. To control wells with cells alone or wells with medium alone, 100 µl of assay medium was added. A standard curve was determined for each plate using standard recombinant human TNF-α (National Institute for Biological Standards and Control) ranging from 0 to 100 IU ml^{-1} and the plates were incubated for 20 h at 37°C with 5% CO_2.

6.2.9.4 Day three

The medium was discarded and the cells stained with 100 µl of filtered 0.5% (w/v) crystal violet in 20% (v/v) methanol in distilled water. This was left for 2 min and the plates washed gently with running tap water. The plates were allowed to dry and the dye dissolved in 100 µl of 20% (v/v) acetic acid in distilled water for 10 min. The optical density was then read in an ELISA plate reader (Dynatech) at 570 nm blanked on the negative controls. The results were expressed as IU ml^{-1} of human TNF-α derived from the standard curve.

6.2.10 Spectrophotometric assay for detection of nitric oxide production

This assay detected the level of nitrite, the stable breakdown product of nitric oxide, in culture supernatants. Greiss reagent 0.3% (w/v) naphthylenediamine dihydrochloride (Sigma) and 1% (w/v) sulfanilamide (Sigma) in 5% (v/v) H_3PO_4 were mixed 1 : 1 immediately prior to use. An equal volume of the reagent was added to 50 µl of culture supernatant and incubated at room temperature for 20 min. The absorbency was read at 570 nm on an ELISA plate reader (Dynatech) which was blanked on a control containing medium alone. A standard curve $NaNO_2$ diluted in DMEM medium alone was included on each plate.

6.2.11 Statistical analyses

The results were analysed by a Student's t-test for paired samples.

6.3 Results

6.3.1 Optimisation of IL-6, IL-β and TNF-β ELISAs

The recombinant standards were used to optimise the dilutions of monoclonal and polyclonal antibodies to their respective cytokines. The average optical density of two duplicate wells was measured and the results of this experiment for IL-6, IL-1β and TNF-β are shown in Figures 6.2a, b and c respectively.

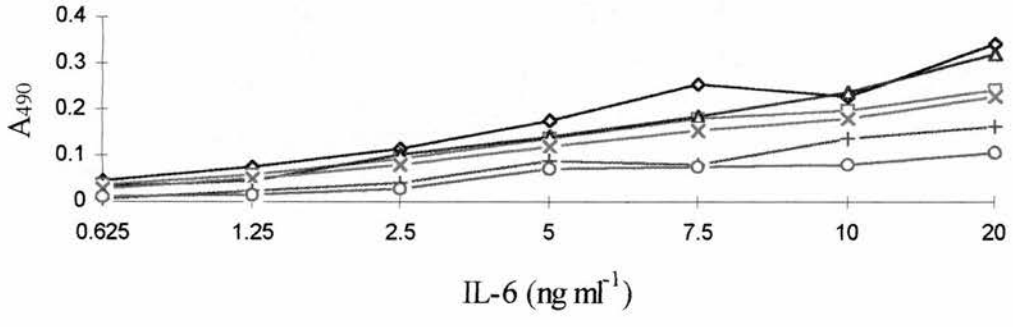
The optimum dilutions for the antibodies to each of the three cytokines were found to be 1 in 500 and 1 in 1,000 for the monoclonal and polyclonal antibodies respectively.

6.3.2 Optimisation of toxin concentration and incubation time for induction of inflammatory mediators

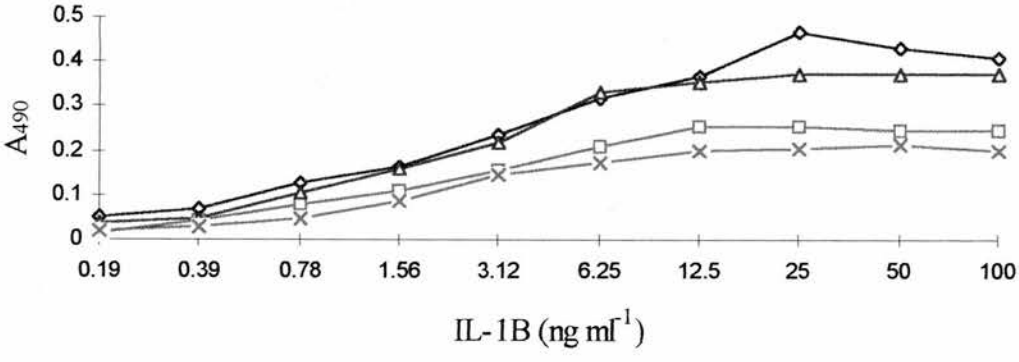
Three buffy coats were tested using TSST-1 concentrations of 0.1, 0.5, 1.0 and 5.0 μg ml^{-1}. Consistent production of TNF-α and IL-6 were observed and the results are shown in Figures 6.3 and 6.4 respectively. There was no consistent production of TNF-β, IL-1β or nitric oxide over the time periods tested. The optimum concentration of TSST-1 in the presence of foetal calf serum was 0.1 μg ml^{-1} and maximum production of TNF-α was observed between 6 and 9 h whilst maximum IL-6 production was observed at 16 h (Figures 6.5 and 6.6).

Figure 6.2 Optimisation of monoclonal and polyclonal dilutions for a) IL-6; b) IL-1β; c) TNF-β ELISAs (m = monoclonal, p = polyclonal)

a) ◊ = m 1/125, p 1/1000; □ = m 1/125, p 1/2000; Δ = 1/500, p 1/1000; × = m 1/500, p 1/2000; + = m 1/1000, p 1/1000; o = m 1/1000, p 1/2000



b) ◊ = m 1/500, p 1/1000; □ = m 1/500, p 1/2000; Δ = m 1/1000, p 1/1000; × = m 1/1000, p 1/2000



c) ◊ = m 1/500, p 1/1000; □ = m 1/500, p 1/2000; Δ = m 1/1000, p 1/1000; × = m 1/1000, p 1/2000

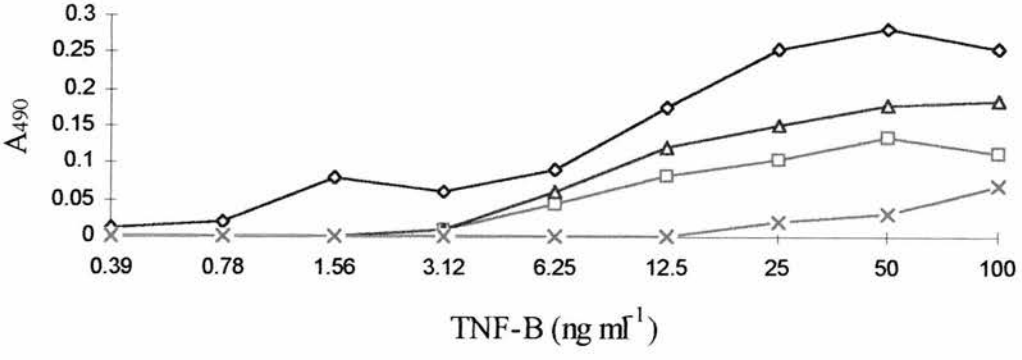


Figure 6.3 Production of TNF-α in response to increasing doses of TSST-1 (n = 3)

(◊ = cells alone, □ = 5.0 μg ml^{-1}, Δ = 1.0 μg ml^{-1}, × = 0.5 μg ml^{-1}, + = 0.1 μg ml^{-1})

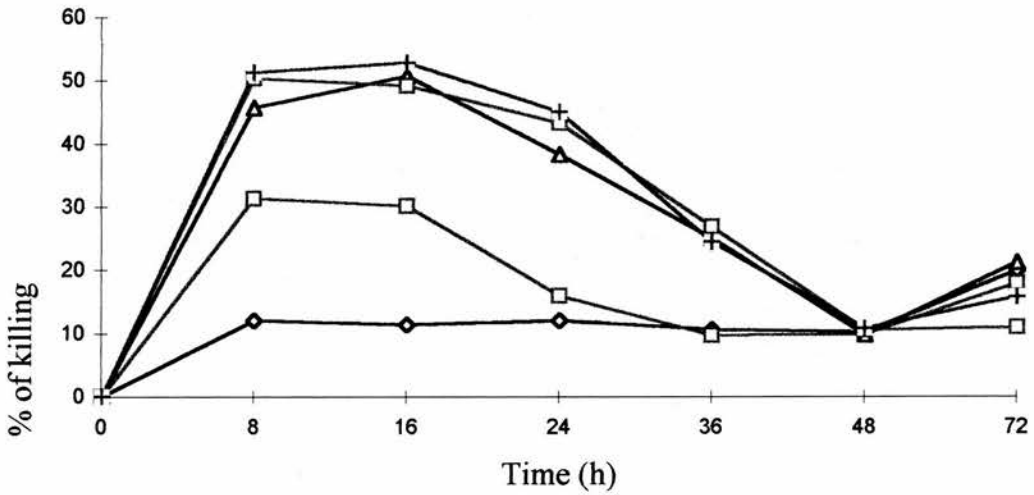


Figure 6.4 Production of IL-6 in response to increasing doses of TSST-1 (n = 3) (◊ = cells alone, □ = 5 μg ml^{-1}, Δ = 1.0 μg ml^{-1}, + = 0.5 μg ml^{-1}, × = 0.1 μg ml^{-1})

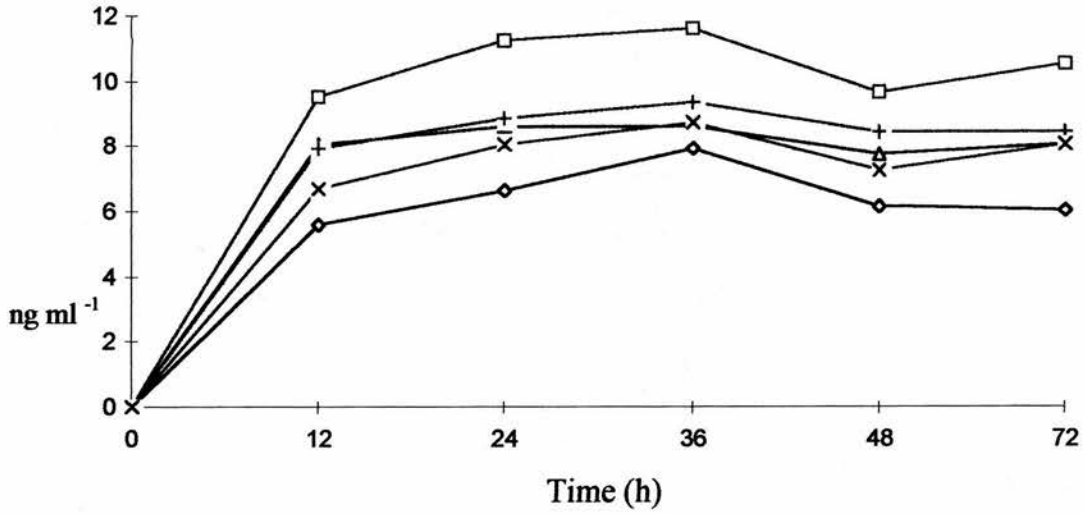


Figure 6.5 Time course for production of TNF-α by human buffy coats in response to 0.1 μg ml^{-1} TSST-1 (n = 9)

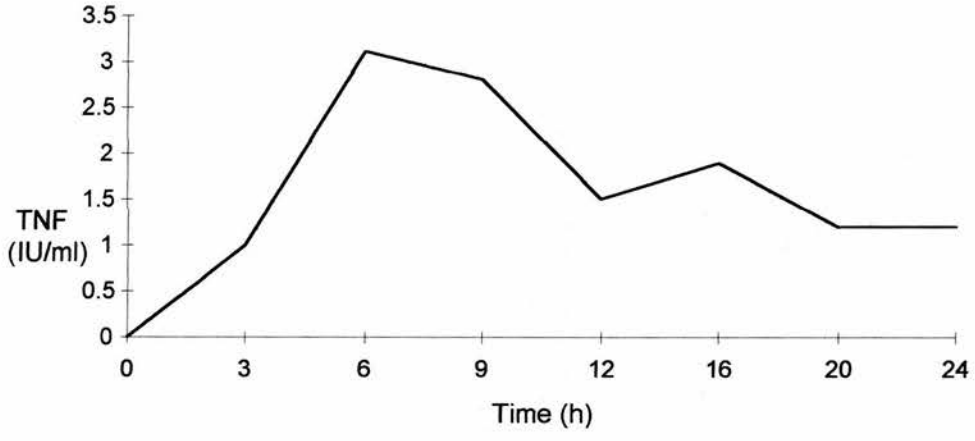
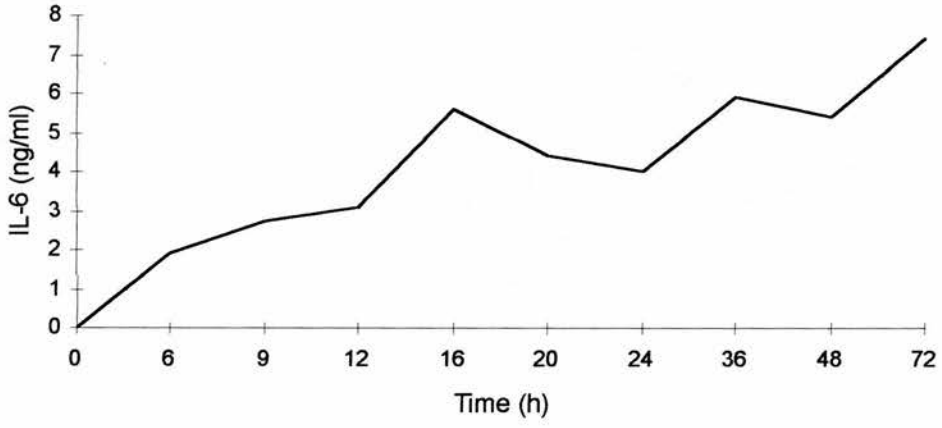


Figure 6.6 Time course for production of IL-6 by human buffy coats in response to 0.1 μg ml^{-1} TSST-1 (n = 7)



6.3.3 Effect of pasteurised human milk and infant formula on induction of inflammatory mediators

The effect of two different dilutions (1 in 10 and 1 in 100) of the pasteurised defatted human milk pool and one infant formula preparation on TNF-α and IL-6 production were investigated. In these experiments, control cells incubated with human milk or infant formula but no toxin, were found to be non-specifically stimulated.

To try to overcome this problem, an experiment was carried out with leukocytes from a group O Rh-negative donor to test three different modifications; 1) no serum added to the system; 2) 5% serum from the donor added to the system; 3) 5% FCS added to the system. For the positive controls of toxin alone, TNF-α production was observed in all conditions tested but IL-6 production was only observed if FCS or serum from the donor was present. Breast milk stimulated production of both TNF-α and IL-6 regardless of the presence or absence of serum.

6.3.4 Effect of synthetic Lewis antigens on TNF-α and IL-6 production in response to TSST-1

6.3.4.1 Optimisation of toxin concentration and incubation time for the Lewis antigen neutralisation studies

Due to non-specific stimulation of the leukocytes by human milk or infant formula, further studies on the role of glycoconjugates in milk were not pursued. The Lewis antigens are also present as free oligosaccharides; therefore, the synthetic antigens were tested in the model system. To reduce further the possibility of non-specific stimulation, homogenous sera of the donor was used to replace FCS in the assay medium. Because of this, the toxin concentrations were re-tested to determine optimal concentrations.

Three buffy coats were tested using TSST-1 at a final concentration of 0.1, 0.5 and 1.0 μg ml^{-1} at 0, 6 and 24 h. Consistent TNF-α and IL-6 production was observed and the results are shown in Figures 6.7 and 6.8 respectively. The optimum concentration of TSST-1 in the presence of individual donor serum was found to be 0.5 μg ml^{-1}.

6.3.4.2 Effect of synthetic Lewis antigens on TNF-α and IL-6 production

Cells incubated with $Lewis^a$ or $Lewis^b$ alone produced similar levels of both TNF-α and IL-6 compared to the negative control cells which were incubated with medium alone (Figures 6.9a and b; Figures 6.10a and b).

Figure 6.7 TNF-α dose response of human buffy coats (n = 3) to different concentrations of TSST-1 with 5% of homogenous donor serum as a replacement for FCS (◊ = no toxin, □ = 0.1 μg ml^{-1}, Δ = 0.5 μg ml^{-1}, x = 1.0 μg ml^{-1})

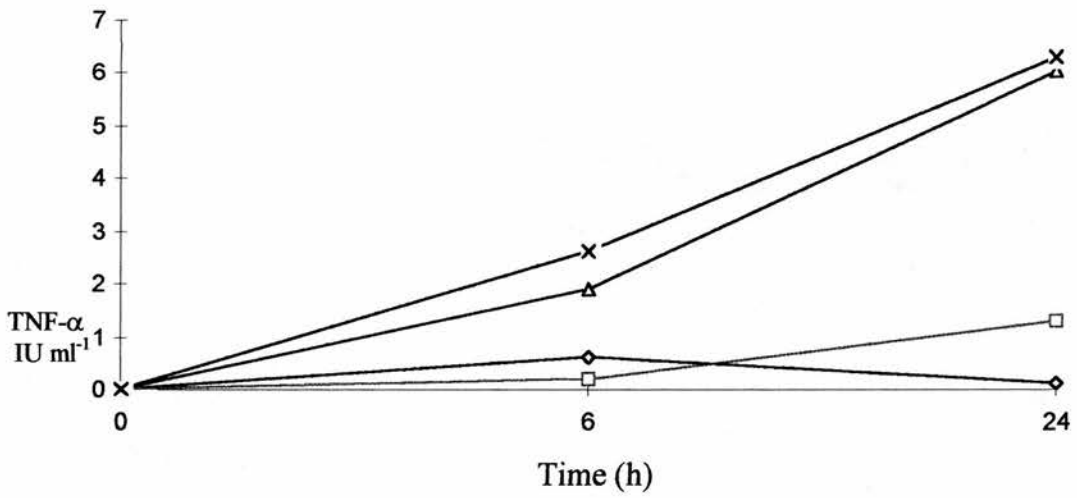


Figure 6.8 IL-6 dose response of human buffy coats (n = 3) to different concentrations of TSST-1 with 5% homogenous donor serum as a replacement for FCS (◊ = no toxin, □ = 0.1 μg ml^{-1}, Δ = 0.5 μg ml^{-1}, x = 1.0 μg ml^{-1})

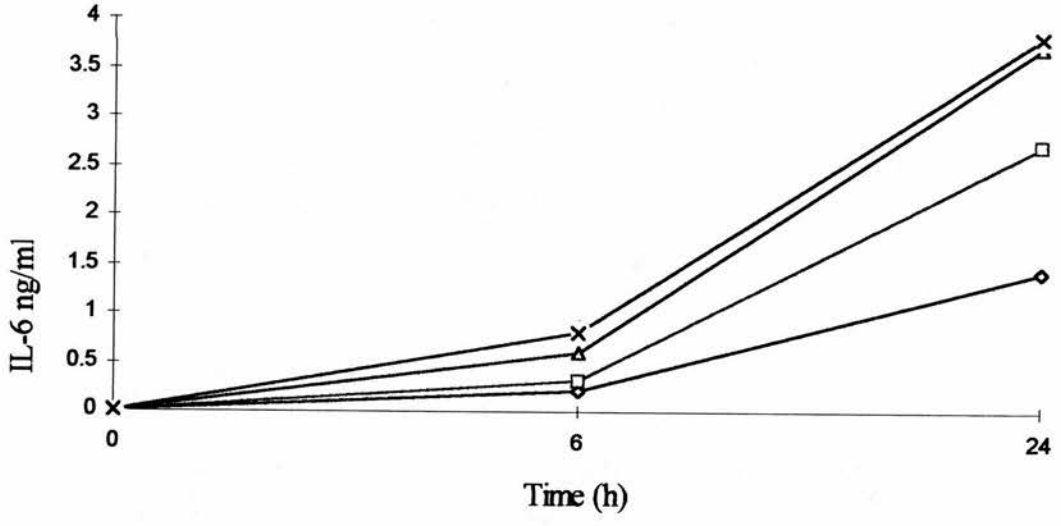
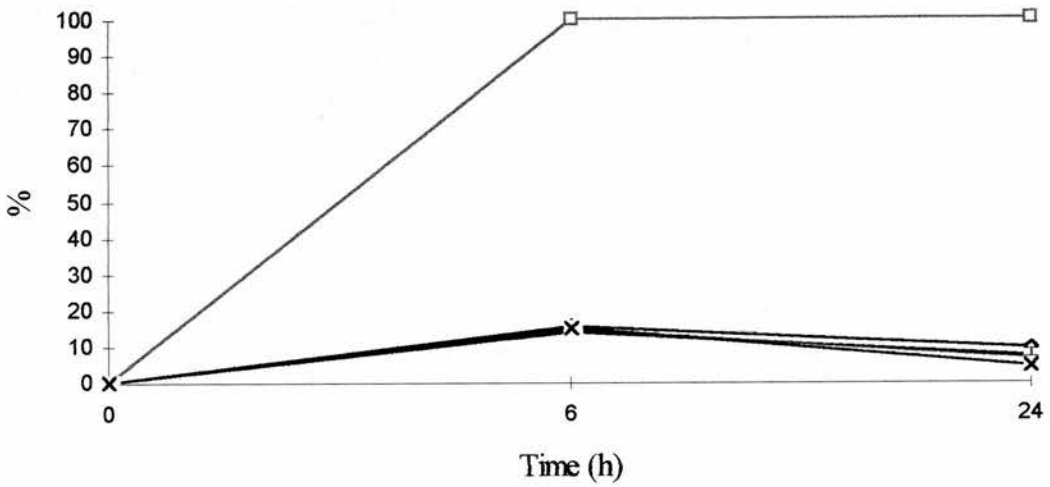


Figure 6.9 Effect of a) Lewis^a and b) Lewis^b on induction of TNF-α (n = 5). (◊ = cells alone; □ = cells plus toxin (0.5 μg ml^{-1}); Δ = cells plus Lewis^a 0.5 μg ml^{-1}; + = cells plus Lewis^a 5 μg ml^{-1}; × = Lewis^a 10 μg ml^{-1})

a)



b)

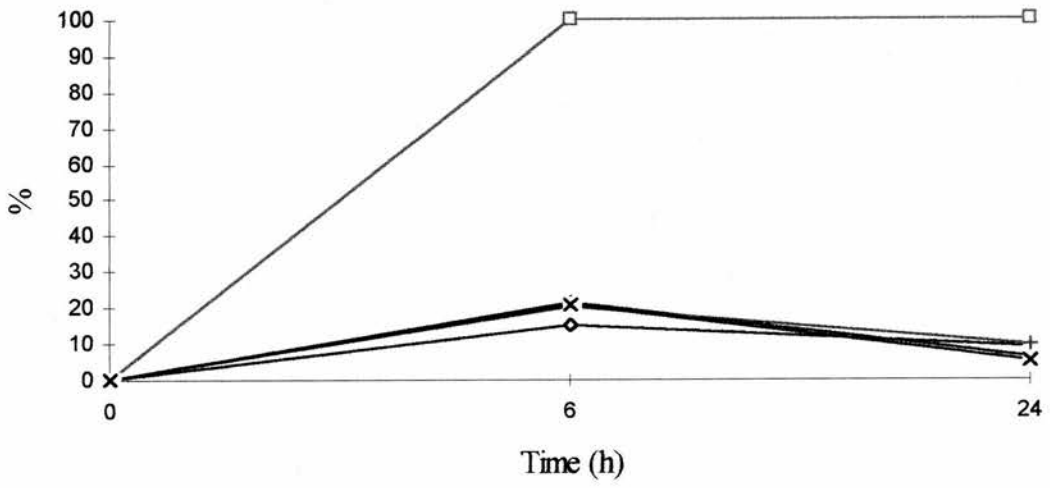
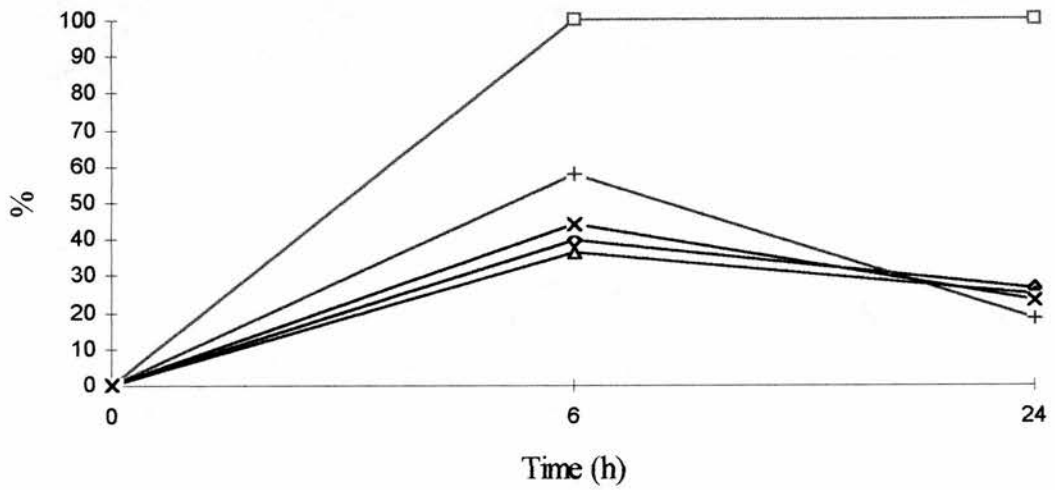
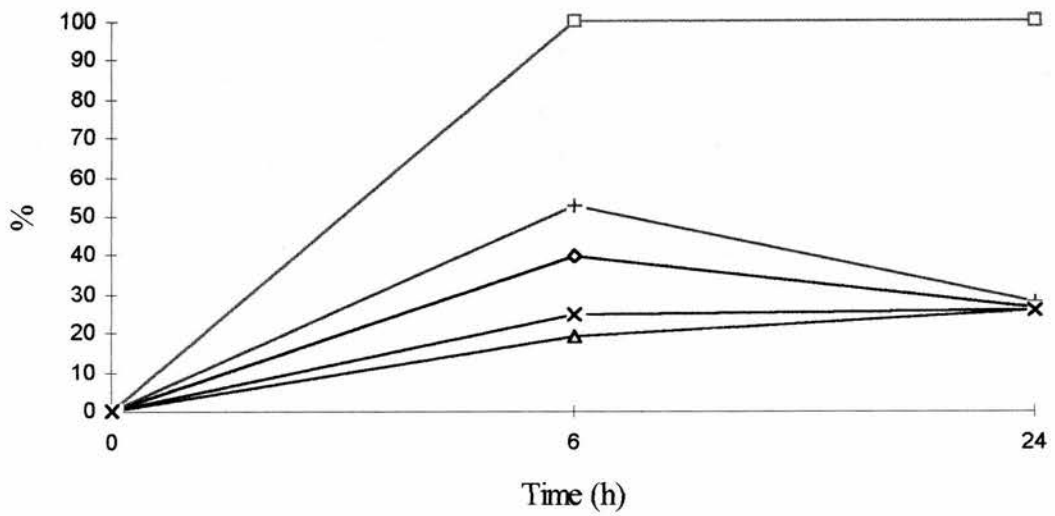


Figure 6.10 Effect of a) Lewis^a and b) Lewis^b on induction of IL-6 (n = 5) (◊ = cells alone; □ = cells plus toxin (0.5 μg ml^{-1}); Δ = cells plus Lewis^a 0.5 μg ml^{-1}; + = cells plus Lewis^a 5 μg ml^{-1}; × = Lewis^a 10 μg ml^{-1})

a)



b)



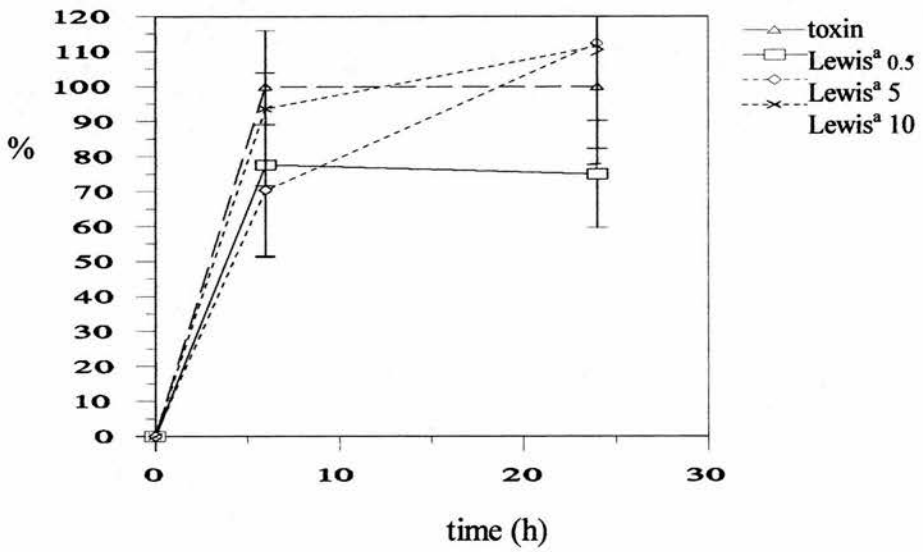
6.3.4.3 Effect of synthetic Lewis antigens on TNF-α and IL-6 production in response to TSST-1

There was no consistent increase or decrease of TNF-α production in response to any concentration of synthetic Lewis^a or Lewis^b antigen tested (Figures 6.11a and b respectively) at either 6 or 24 h. The lowest levels of TNF-α were observed for toxin incubated with 0.5 μg ml^{-1} of the Lewis antigen and the level of TNF-α appeared to increase with increasing amounts of the Lewis antigen.

There was a significant decrease in IL-6 production in response to TSST-1 for all concentrations of synthetic Lewis^a tested at both 6 h (0.5 μg ml^{-1} p=0.003; 5 μg ml^{-1} p=0.049; 10 μg ml^{-1} p=0.024) and 24 h (0.5 μg ml^{-1} p=0.037; 5 μg ml^{-1} p=0.046; 10 μg ml^{-1} p=0.041) (Figure 6.12a). There was a significant decrease in IL-6 production at 6 h by synthetic Lewis^b at a concentration of 5 μg ml^{-1} (p=0.031); however, a significant increase in IL-6 production was observed at 24 h (p=0.020) by synthetic Lewis^b at a concentration of 10 μg ml^{-1} (Figure 6.12b). As with the results for TNF, lowest levels of IL-6 were observed for 0.5 μg ml^{-1} of the antigens.

Figure 6.11 Effect on TNF-α production in response to TSST-1 in the presence of the synthetic Lewis antigens (n = 5) a) Lewis^a b) Lewis^b

a)



b)

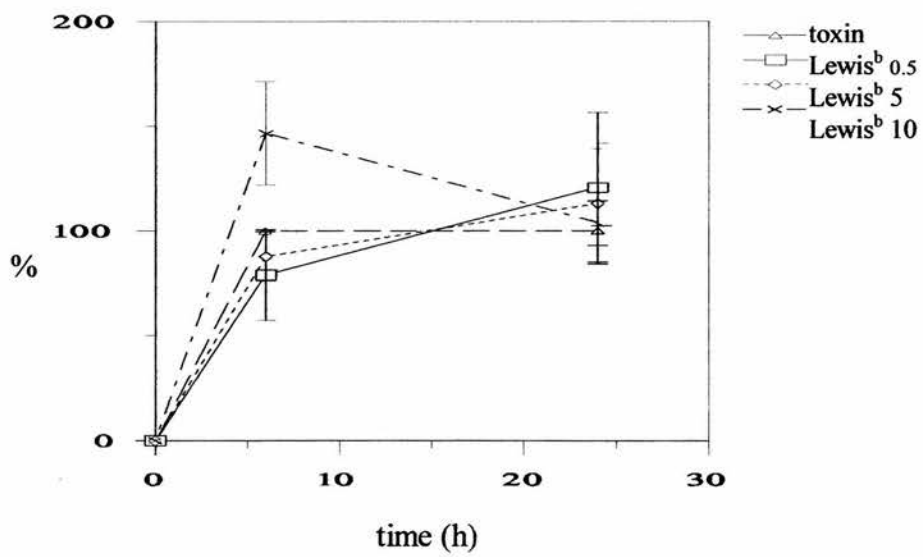
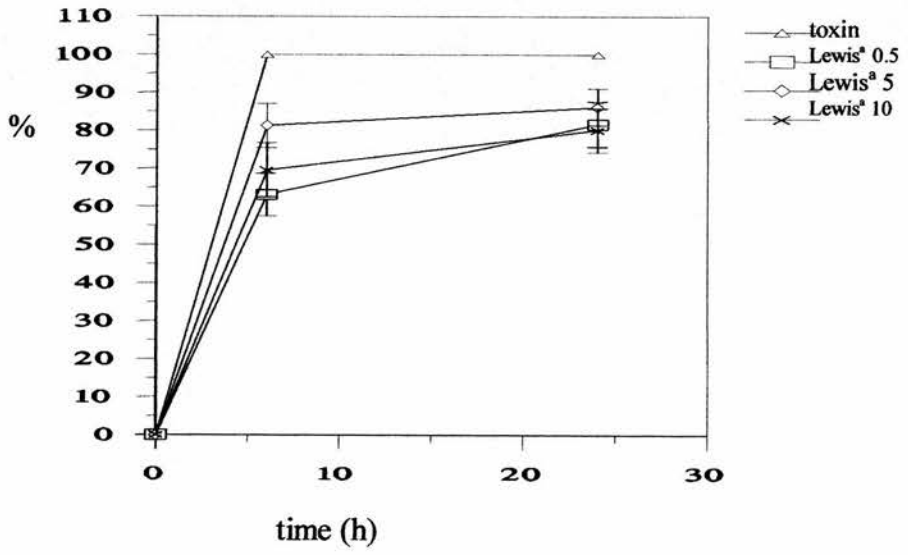
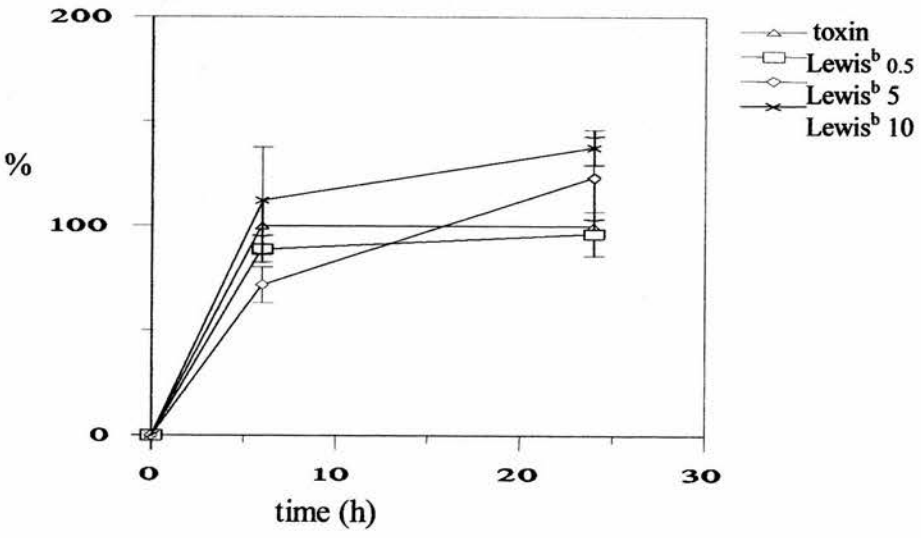


Figure 6.12 Effect on IL-6 production in response to TSST-1 in the presence of the synthetic Lewis antigens (n = 5) a) Lewis^a b) Lewis^b

a)



b)



6.3.5 Effect of cortisol on TNF-α and IL-6 production in response to TSST-1

6.3.5.1 Effect of cortisol on TNF-α and IL-6 production

Preliminary studies examined the effect of cortisol concentrations (0.62 - 25 μg dl^{-1}) on induction of TNF-α and IL-6 from buffy coat cells. The results for concentrations ≤ 5 μg dl^{-1} and > 5 μg dl^{-1} are shown in Figures 6.13 (n = 9) and 6.14 (n = 7). Cells incubated with cortisol produced levels of both cytokines similar to the negative control cells which were incubated with medium alone.

6.3.5.2 Effect of cortisol on TNF-α and IL-6 production in response to TSST-1

Concentrations of cortisol ranging from 0.62 to 25 μg dl^{-1} were used in these experiments (n = 9 for TNF-α and n = 7 for IL-6) to assess their effects on leukocyte responses to 0.1 μg ml^{-1}. The results have been divided in to two groups. The first group included concentrations of cortisol less than or equal to 5 μg dl^{-1} which are representative of levels present in infants at night after the development of circadian rhythm. The second group included concentrations of cortisol greater than 5 μg dl^{-1} representative of concentrations of cortisol in infants during the day or at night prior to the development of the circadian rhythm.

Concentrations of cortisol less than or equal to 5 μg dl^{-1} did not consistently increase or decrease TNF-α production over the time period examined. There was a significant increase in TNF-α at 20 h which was not affected by any concentration of cortisol tested. Concentrations of cortisol greater than 5 μg dl^{-1} significantly

decreased TNF-α production at 12 h ($p = 0.005$) (Figure 6.15). Concentrations of cortisol less than or equal to 5 μg dl^{-1} did not significantly increase or decrease IL-6 production; however, concentrations of cortisol greater than 5 μg dl^{-1} significantly decreased IL-6 production in response to TSST-1 at 12 h ($p = 0.019$) and 16 h ($p = 0.010$) (Figure 6.16). IL-6 levels observed with the higher cortisol levels were consistently lower than those obtained with ≤ 5 μg dl^{-1}.

Figure 6.13 Effect of cortisol alone on TNF-α production from human buffy coats (n = 9) (DMEM = ♦, TSST-1 = x, cortisol (≤ 5 μg dl^{-1}) = ■, cortisol (> 5 μg dl^{-1}) = ▲)

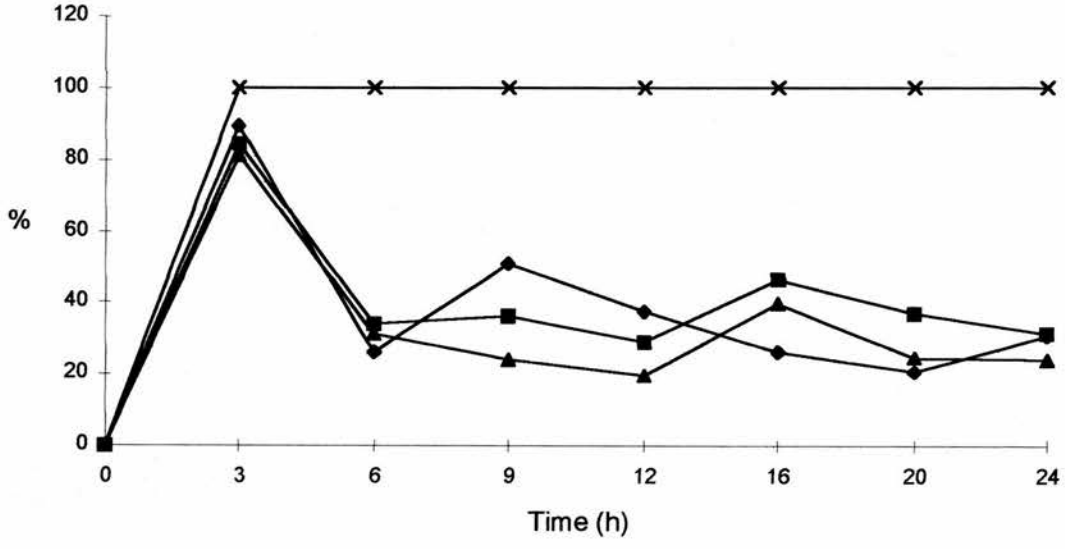


Figure 6.14 Effect of cortisol alone on IL-6 production from human buffy coats (n = 7) (DMEM = ♦, TSST-1 = x, cortisol (≤ 5 μg dl^{-1}) = ■, cortisol (> 5 μg dl^{-1}) = ▲)

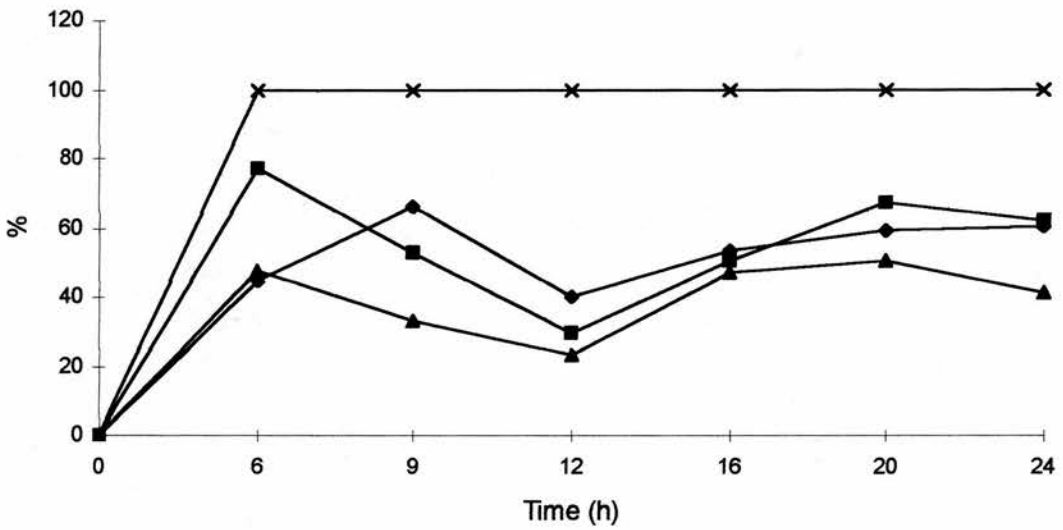


Figure 6.15 Effect of cortisol on TNF-α production from human buffy coats stimulated with TSST-1 (n = 9) (TSST-1 = Δ, cortisol (≤ 5 μg dl^{-1}) = □, cortisol (> 5 g dl^{-1}) = ◊)

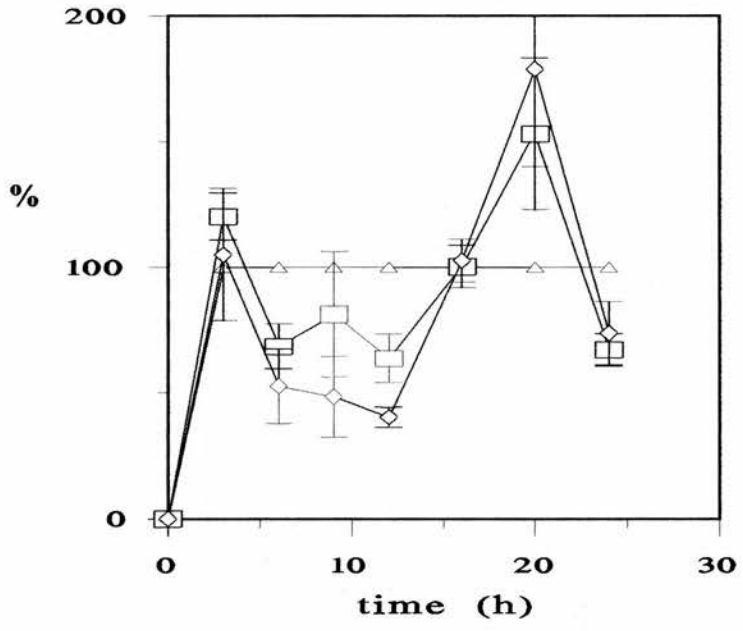
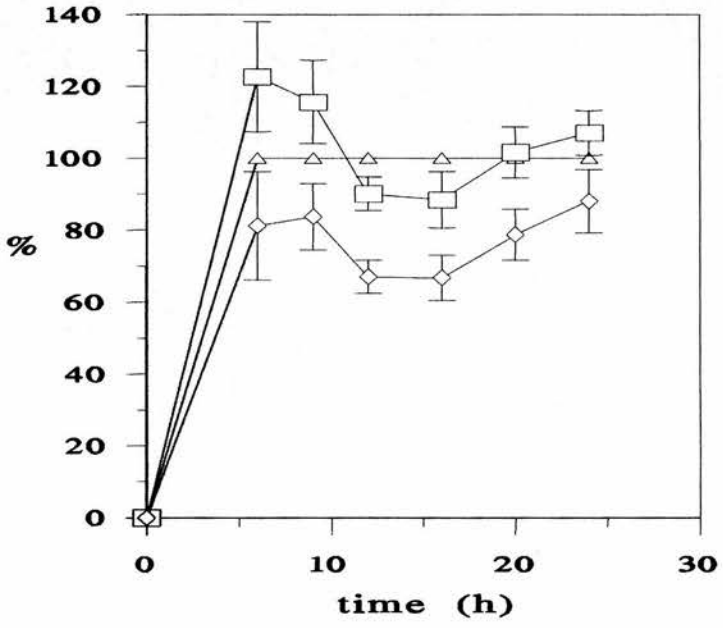


Figure 6.16 Effect of cortisol on IL-6 production from human buffy coats stimulated with TSST-1 (n = 7) (TSST-1 = Δ, cortisol (≤ 5 μg dl^{-1}) = □, cortisol (> 5 g dl^{-1}) = ◊)



6.4 Discussion

6.4.1 Lewis antigen studies

Non-specific cytokine stimulation of human leukocytes in response to breast milk or infant formula in the absence of toxin meant that the model could not be used to test for neutralisation of the toxin. One problem which might affect the studies involving breast milk is that breast milk contains IL-6 and TNF-α [Rudloff *et al.*, 1991, Saito *et al.*, 1991]; however, no TNF-α or IL-6 were detected at time 0 h which might be due to the effect of pasteurisation. Concentrations of these cytokines are higher in colostrum compared to mature milk, and other work has suggested that IL-6 present in human milk is associated with local IgA production in the breast [Saito *et al.*, 1991]. Another problem which might affect studies with infant formula is that it has been previously suggested that some infant formulas might stimulate human immune responses [Lambert *et al.*, 1995].

Since both breast milk and infant formula contain Lewis^a and Lewis^b antigens, the model was used to test the effects of these on the ability of TSST-1 to induce TNF-α and IL-6 production. Studies have shown that toxins can bind to Lewis antigens expressed on monocytes and pre-treatment of monocytes with monoclonal anti-Lewis^a antibodies inhibited binding of TSST-1 and SEC [Essery *et al.*, 1994; Essery, 1997].

Cleary *et al*; [1983] suggested that a carbohydrate molecule present in human milk could protect suckling mice against the *E. coli* heat-stable enterotoxin and later work showed that this molecule was a fucosyl containing oligosaccharide [Newburg *et al.*, 1990]. Oligosaccharides from human milk have also been found to inhibit binding and activity of the *E. coli* heat stable enterotoxin to human T84 intestinal cells [Crane *et al.*, 1994]. Oligosaccharide-derivatized dendrimers (a hyper-branched polymer that radiates from a central core) have also been found to inhibit adherence of cholera toxin and the heat-labile enterotoxin of *E. coli* to cell-surface monoganglioside gal(β1-3)galNAc(β1-4)[sialic acid(α2-3)](gal(β1-4)glcβ1-ceramide (GM1) which is present on intestinal epithelial cells [Thompson *et al.*, 1998].

The oligosaccharides alone did not induce either TNF-α or IL-6 production. For IL-6 at both 6 and 24 h, a significant decrease in IL-6 production in response to TSST-1 was observed for all three concentrations of Lewis^a tested. A significant decrease in IL-6 production was observed at 6 h for 5 μg ml^{-1} of Lewis^b; however, the significant increase in IL-6 production at 24 h in response to TSST-1 and Lewis^b 10 μg ml^{-1} was not expected. Work carried out by other groups using fibroblast cells has found that there are two different pathways of IL-6 production. One is regulated by activation of protein kinase C [Sehgal *et al.*, 1987] and the second is controlled by activation of the cAMP dependent signalling pathway [Zhang *et al.*, 1988]. It might be that the Lewis^a and Lewis^b antigens act to inhibit two independent pathways of IL-6 production.

The consistent results with Lewis^a might reflect the fact that all but about 3 - 5% of individuals express this antigen; however, the quantities on cells can vary widely. Non-secretors usually have high levels of Lewis^a and no Lewis^b but secretors can also express high levels of Lewis^a. This depends on the efficacy of the fucosyl transferase coded for by the secretor gene. It might be that non-secretors produce inflammatory mediators in response to high concentrations of Lewis^b. If the donors used in the buffy coat experiments were non-secretors, this might explain why there was increased IL-6 production in response to high concentrations of Lewis^b.

There was no consistent effect of either Lewis^a or Lewis^b antigens on TNF-α production. It might be that TSST-1 induces production of TNF-α and IL-6 by two different pathways.

To investigate this enhancement of IL-6 production, these experiments could be repeated in relation to quantitative assessment of the level of Lewis antigens on the leukocytes. For example leukocytes from non-secretors who express only Lewis^a might be stimulated by the higher concentration of Lewis^b whereas a secretor might not be stimulated by either Lewis^a or Lewis^b. It would also be useful to carry out these experiments using a slightly modified protocol in which TSST-1 was initially incubated with the appropriate concentration of Lewis^a or Lewis^b antigen for 2 h at 37°C before addition to the cells and TNF-α and IL-6 production at 6 and 24 h measured. Lower concentrations of the antigen also need to be examined as the

lower levels of TNF-α and IL-6 were noted for the lowest concentrations of the Lewis antigens tested.

6.4.2 Night-time occurrence of SIDS

The night-time prevalence of SIDS deaths prompted studies on the physiological development of infants to explain the association with sleep and the age range during which the majority of these deaths occur (2 – 4 months). These studies identified significant decreases in night-time cortisol levels closely following the switch to lower night-time body temperature associated with development of circadian rhythm. These observations could be highly relevant to the hypothesis that inflammatory reactions triggered by infection are involved in the events resulting in death. The range of night-time cortisol levels found in infants after the switch to the adult-like night-time temperature rhythm is 0.1 – 7.25 μg dl^{-1}. The range of cortisol levels tested (0.5 - 5 μg dl^{-1}) were within this range and were insufficient to control inflammatory responses induced by TSST-1. Levels as high as 10 μg dl^{-1} were not found to have any consistent effect on induction of either IL-6 or TNF-α [Al Madani, 1999].

In various tissues, glucocorticoids have been found to regulate negatively IL-6 gene expression through a repressive mechanism of IL-6 promoter activity [Takeshita *et al.*, 1996]. Glucocorticoids have been found to do this by repressing the activity of the transcriptional enhancer NF-κB by a dual mechanism involving protein-protein

interactions between NF-κB and the glucocorticoid receptor and induction of IκBα which terminates the NF-κB response [Wissink *et al.*, 1998].

6.4.3 Peak incidence of SIDS in the 2 – 4 month age range

During the 2 – 4 month age range, decreasing levels of maternal antibodies against bacteria and viruses result in infants being more reliant on their inflammatory responses to deal with infection. Significant inflammatory responses might be quite common among infants; however, if the events occur during a period in which the ability to control these responses is reduced, the result might be SIDS or a "near miss" episode. Indirect evidence that younger infants have reduced or better controlled inflammatory responses to micro-organisms comes from a large study carried out in conjunction with the change in immunisation schedules in Britain. One of the unexpected findings associated with the change was the significant decrease in adverse reactions (fever or local inflammatory reactions) to the diphtheria, pertussis and tetanus vaccine among infants immunised at 2 months compared with those immunised at 3 months. This was found for both acellular and whole cell pertussis vaccines [Miller *et al.*, 1997]. If, as the work on night-time levels of cortisol suggests, younger infants are better able to deal with inflammatory mediators because of higher cortisol levels, the new immunisation schedule is helping to increase levels of antibodies against bacterial toxins during a period in which infants are better able to handle inflammatory responses.

Wailoo and colleagues found that infants who share many of the risk factors associated with SIDS remain in the "immature" developmental stage longer than those with few risk factors. They suggested that this prolonged developmental stage might contribute to susceptibility to SIDS. Alternatively, infants who remain in this developmental stage are better able to control inflammatory responses and benefit from the extra time to produce active immunity through vaccination or natural exposure. An example is the observation that Asian infants remain in the immature stage longer than white infants [Petersen *et al.*, 1994] although the majority are breast fed. Poverty is strongly associated with SIDS [Blair *et al.*, 1996; Brooke *et al.*, 1997]. In Britain, studies carried out in Birmingham and across the UK found that most of the Asian families were classified as socially deprived [Farooqi *et al.*, 1995; Church, 1996]; however, Asian families had a lower incidence of SIDS and deaths due to respiratory infections compared to white families [Balarajan *et al.*, 1989].

6.4.4 Conclusions

Due to non-specific stimulation, it was not possible to determine whether or not breast milk or infant formula could reduce pro-inflammatory cytokine production in response to TSST-1. Serum IgG antibodies to TSST-1 and SEC were found to reduce induction of inflammatory mediators by these toxins [Al Madani, 1999]. The IgA antibodies found in human milk to the toxins [Chapter 5] would be expected to neutralise the toxins on mucosal surfaces. Lewis antigens are found in breast milk

and in these studies Lewis^{a} (but not Lewis^{b}) significantly and consistently decreased IL-6 production in response to TSST-1.

The findings of this chapter also indicate that cortisol levels observed during both the day and at night prior to development of the circadian rhythm would contribute to control of pro-inflammatory cytokines; however, levels observed at night after development of the circadian rhythm pattern might not be sufficient to control production of TNF-α or IL-6. If this switch to circadian rhythm occurs early when maternal antibodies are still present or an infant is receiving IgA in breast milk, or later when immunisation or natural exposure has induced protective antibodies, inflammatory responses to common infections would be better controlled. If the switch occurs during the period in which maternal antibodies are very low, an infant is formula fed or the infant has not produced its own active immunity, uncontrolled inflammatory responses produced against micro-organisms or their products might precipitate the series of events leading to a SIDS death.

Chapter Seven

Detection of TNF-α and IL-6 gene expression by reverse transcriptase – PCR (RT-PCR)

7.1 Introduction

Bacterial toxins and the inflammatory mediators they induce have been implicated in the mechanism of a SIDS death. Although toxins have been detected in tissues taken from some SIDS infants and inflammatory mediators have been observed, there are no studies which have examined specimens for both the toxins and for evidence of the inflammatory responses.

Blood samples taken at autopsy often arrive too late for accurate screening for cytokines. An alternative approach would be to examine tissues for gene expression. This would need to be correlated to the interval between death and autopsy and the method of storage.

Human leukocytes consistently produced the pro-inflammatory cytokines TNF-α and IL-6 [Chapter 6] in response to TSST-1. The cells from these experiments had been stored at -70°C. The aim of this part of the project was to carry out preliminary studies with the stored cells to develop a reverse-transcriptase polymerase chain reaction (RT-PCR) technique to detect messenger RNA (mRNA) for TNF-α and IL-6. A positive β-actin control was included. This gene is constitutively expressed and mRNA should be detectable at all times.

For detection of changes of gene expression by RT-PCR, expression must be controlled at the level of transcription. There is evidence from nuclear run-on transcription assays that TSST-1 can cause transcriptional activation of TNF-α gene

expression in human peripheral blood monocytes and the monocytic cell culture line, THP-1. These studies found that non-stimulated primary human monocytes have very low levels of TNF-α mRNA but upon addition of TSST-1, there is *de novo* gene transcription and a rapid increase in steady state TNF-α mRNA levels [Trede *et al.*, 1991]. Further studies indicated that TNF-α gene expression is tightly regulated at both the transcriptional and translational levels by MHC-class II ligands. Upon TSST-1 binding to MHC-class II molecules, there is a fast peak transient induction of TNF-α mRNA and nuclear run-on assays indicated that there was a 4-fold increase in the rate of transcription which did not require *de novo* protein synthesis. Induction of TNF-α transcripts in monocytes by TSST-1 has been found to be dose-dependent with a plateau of TNF-α expression reached at 1 $\mu g\ ml^{-1}$ of TSST-1 [Espel *et al.*, 1996].

TSST-1 has been found to induce rapid activation of the transcriptional enhancer NF-κB [Trede *et al.*, 1993]. One possible method of transcriptional activation of TNF-α by superantigens was investigated by studies involving THP-1 cells and SEA. In these experiments, SEA induced NF-κB activation and NF-κB bound strongly to the TNF-α promoter at the κ3 site which is the most proximal of the three putative NF-κB sites (Figure 7.1). This results in transcriptional activation of the TNF-α gene [Trede *et al.*, 1995]. Induction of TNF-α gene transcription in monocytes by TSST-1 has also been found to involve activation of protein kinase C [Trede *et al.*, 1994].

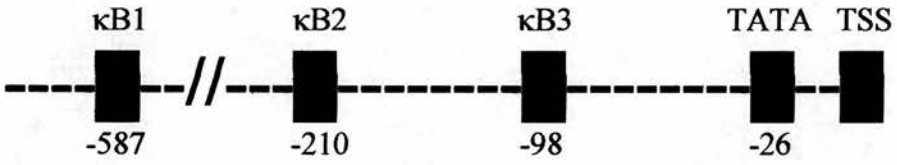
IL-6 has a central role in the host defence mechanism and requires an "early" response. In accordance with this, there is evidence that IL-6 is transcriptionally induced in a variety of tissues in response to a variety of substances which trigger inflammation. These include bacterial endotoxins, virus infections, tissue damage inducing agents, inflammation associated cytokines such as IL-1β, TNF-α and interferons [Faggioli *et al.*, 1997].

One potential problem with the application of these studies to SIDS infants is that many tissues taken from these infants at autopsy are stored in formalin which is not a good fixative for nucleic acids.

The aims of this part of the study were:

1) to develop an RT-PCR technique to detect TNF-α and IL-6 gene expression in the stored leukocytes which had been stimulated with TSST-1;
2) to determine the effect of freezing and formalin fixation on mRNA detection.

Figure 7.1 The human TNF-α promoter and the κ3 site [Trede *et al.*, 1995].



7.2 Materials and methods

7.2.1 Extraction of RNA

Two different RNA extraction protocols were used and are described in 7.2.1.1 and 7.2.1.2. For RNA extraction, all solutions were prepared using Millipore pure water which had been treated with 0.1% diethyl pyrocarbonate (DEPC) (Sigma). To prepare the water, 0.1% DEPC was added to the water, incubated overnight at 37°C and then autoclaved to remove all traces of DEPC. All glassware used was incubated overnight at 37°C with a 0.1% DEPC solution and then autoclaved. All chemicals used had been tested for the presence of any RNases, DNases and proteases and were certified free.

RNA was extracted from leukocytes (2 x 10^6 ml^{-1}) which had been used in the model system developed in Chapter 6. These cells had been stored in freezing medium (100 ml contains: 50 ml FCS; 10 ml dimethylsulphoxide (Sigma); 40 ml RPMI medium) at -70°C. Before RNA extraction was carried out, the cells were thawed, washed in DMEM medium (stored at 4°C) by centrifugation at 250 x *g* for 15 min at 4°C to remove all traces of the freezing medium and the supernatant was carefully removed without disturbing the cell pellet. The cells were stored on ice.

7.2.1.1 RNA extraction by guanidinium thiocyante phenol-chloroform extraction

The method of Chomczynski *et al*; [1987] was adapted for these experiments. All solutions were stored on ice before use.

Denaturing solution D (200 µl) containing 4M guanidine-thiocyanate (Sigma), 25 mM sodium citrate (pH 7), 0.5% (w/v) sarcosyl and 0.1 M 2-mercaptoethanol (Sigma) was added to the cells. The cells were resuspended and transferred to 2.0 ml Eppendorf tubes (Greiner). Sodium acetate (2M, pH 4) (20 µl) (Sigma) and phenol-chloroform (200 µl) (Sigma) were added and the contents mixed by inversion after each addition. The tubes were shaken vigorously for 10 sec and stored on ice for 15 min. The tubes were centrifuged at 10,000 x *g* for 20 min at 4°C and the upper aqueous layer containing the RNA was transferred to a clean Eppendorf tube. This step was then repeated. Isopropanol (200 µl) was added and the tube stored at –20°C for 1 h. The RNA was precipitated by centrifugation at 10,000 x *g* for 20 min at 4°C. The pellet was resuspended in 60 µl of solution D and 60 µl of isopropanol and stored at -20°C for 1 h. After centrifugation at 10,000 x *g* for 20 min at 4°C, the pellet was washed with 200 µl of 75% ethanol and centrifuged as described. Excess ethanol was removed and the pellet was allowed to dry. The pellet was resuspended in 50 µl of DEPC-treated water and stored at -70°C until use.

7.2.1.2 Extraction of RNA by the Qiagen RNeasy Mini Kit

The RNeasy mini protocol for the isolation of total RNA from animal cells was used. All steps were carried out at room temperature. All solutions and RNase free water used were contained in this kit and the method was carried out as follows.

The cells were prepared as described and the cell pellet loosened by flicking the tube. To disrupt the cells, Buffer RLT (350 µl) containing β-mercaptoethanol (Sigma) (10 µl per 1 ml of Buffer RLT) were added and the mixture pipetted until no cell clumps were visible. To homogenise the samples, the lysate was added to a QIAshredder column (Qiagen) and centrifuged for 2 min at 8,000 x *g*. One volume (350 µl) of 70% ethanol was added to the homogenised lysate and mixed by pipetting. The lysate (700 µl) was added to an RNeasy spin column sitting in a 2.0 ml collection tube and centrifuged for 15 sec at 8,000 x *g*. The flow-through was discarded. Buffer RW1 (700 µl) was added to the RNeasy column for 5 min and then the tube centrifuged for 15 sec at 8,000 x *g*. The flow-through and collection tube were discarded. The RNeasy spin column was transferred to a clean 2.0 ml collection tube and 500 µl of Buffer RPE was added to the RNeasy column. After centrifugation for 15 sec at 8,000 x *g*, the flow-through was discarded and a further 500 µl of Buffer RPE was added to the column. To completely dry the membrane, the column was centrifuged for 2 min at 8,000 x *g*. The column was transferred to a new 1.5 ml collection tube and 30 µl of RNase-free water added to the membrane. To elute the RNA, the column was centrifuged for 1 min at 8,000 x *g*. The RNA preparation was stored at -70°C until use.

7.2.3 Quantitation of RNA

This was carried out by UV spectrophotometry. The spectrophotometer was set at 260 nm and blanked using a water alone control. 5 µl of the RNA preparation was added to 995 µl of distilled water and the A_{260} determined. The concentration of

RNA in the preparation was calculated using the standard value that RNA at a concentration of 40 μg ml^{-1} has an OD of 1 [Sambrook *et al.*, 1989].

7.2.4 DNase digestion of RNA preparations and RNA cleanup

7.2.4.1 DNase digestion of RNA preparations

The DNase, DNase reaction buffer (supplied as a 10 times concentrate) and stopping solution were obtained from Promega. The reaction mixture consisted of the following: 22.5 μl of the RNA preparation; 1.5 μl of RNase inhibitor; 3 μl of 10 x DNase reaction buffer; 3 μl of DNase (final volume of 30 μl). After mixing, the reaction mixture was overlaid with 50 μl of mineral oil and incubated for 30 min at 37°C; 3 μl of stopping solution was added and the mixture incubated at 65°C for 10 min.

7.2.4.2 RNA cleanup protocol

The RNeasy Mini Kit (Qiagen) was used to purify the RNA from the components of the DNase digestion. All solutions and RNase free water used were contained in this kit and the method was carried out as follows.

RNase free water (70 μl) was added to the DNase digested preparation. Buffer RLT (350 μl plus 10 μl of β-mercaptoethanol per 1 ml of Buffer RLT) was added and the samples mixed thoroughly. Ethanol (70% v/v) (250 μl) was added and the mixture mixed well by pipetting. The samples (700 μl) were applied to the RNeasy spin

column and the column centrifuged for 15 sec in a bench top centrifuge at 8,000 x *g*. The flow through and collection tube were discarded and the column added to a new collection tube. Buffer RPE (500 µl) was added and the column centrifuged for 15 sec at 8,000 x *g*. The flow through was discarded and 500 µl of buffer RPE added. The column was centrifuged for 2 min at 8,000 x g. The RNA was eluted by addition of 30 µl RNase free water and the column centrifuged at 8,000 x *g* for 1 min. The digested RNA was stored at -70°C.

7.2.5 RT-PCR for TNF-α, IL-6 and β-actin

The RT-PCR core kit (Perkin-Elmer) was used according to the provided instructions. After thawing, all components were vortexed and stored on ice until use. A positive control of β-actin and negative controls in which no RNA or no reverse transcriptase had been added, were included.

For reverse transcription (RT), a master mix was prepared and contained the following in each reaction: 4 µl of 25 mM $MgCl_2$ solution; 2 µl of 10 x PCR buffer II (supplied at 10 times the working concentration); 1 µl of DEPC-treated water; 2 µl dGTP (10 mM); 2 µl dATP (10 mM); 2 µl dTTP (10 mM); 2 µl dCTP (10 mM); 1 µl of oligo d(T)16 (50 µM); 1 µl of RNase inhibitor (20 U μl^{-1}); 1 µl of Murine Leukemia Virus Reverse Transcriptase (MuLV RT) (50 U μl^{-1}). The master mix (18 µl) was aliquoted into individual thin-walled PCR tubes (Greiner) and 2 µl of the RNA extract was added to give a final reaction volume of 20 µl. The addition of DEPC water could be omitted if 3 µl of the RNA extract was to be added. The

reaction was overlaid with 50 µl of mineral oil (Sigma) and the RT conditions used were: 42°C for 15 min; 99°C for 5 min; 15°C for 5 min. The PCR machine used was a THC-2 model (Tecan).

For PCR, a master mix was prepared which contained per reaction: 4 µl of 25 mM $MgCl_2$ solution; 8 µl of 10 x PCR buffer II; 65.5 µl of sterile Millipore pure water; 0.5 µl of AmpliTaq DNA polymerase (5 U μl^{-1}). This mixture (78 µl) was aliquoted into individual thin-walled tubes and 2 µl of the appropriate specific primer added. The RT (20 µl) reaction was added to give a final volume of 100 µl. After mixing, the reaction was overlaid with 50 µl of mineral oil and the PCR conditions used were: 94°C for 5 min; 60°C for 5 min. These were followed by 25 cycles for IL-6 and β–actin and 35 cycles for TNF-α of 72°C for 1 min 30 sec, 94°C for 45 sec and 60°C for 45 sec. This was followed by a final extension of 72°C for 10 min.

Table 7.1 Sense and antisense primer sequences (Stratagene)

Primer	**Predicted product size**	**Sense or Antisense**	**Sequence**
TNF-α	355 base pairs	Sense	174 CGGGACGTGGAGCTGGCCGAGGAG 197
		Antisense	504 CACCAGCTGGTTATCTCTCAGCTC 528
IL-6	628 base pairs	Sense	34 ATGAACTCCTTCTCCACAAGCGC 56
		Antisense	640 GAAGAGCCCTCAGGCTGGACTG 661
β-actin	661 base pairs	Sense	1038 TGACGGGGTCACCCACACTGTGCCCATCTA 1067
		Antisense	1876 CTAGAAGCATTTGCGGTGGACGATGGAGGG 1905

7.2.6 Primer sequences

TNF-α, IL-6 and β-actin primers were all obtained from (Stratagene). The primers were reconstituted in 100 μl of 5 mM Tris.HCl and 0.1 mM EDTA solution and stored in 20 μl aliquots at -20°C. The sequences are shown in Table 7.1. The primers have been designed to span an intron-exon boundary and this should ensure that an mRNA derived PCR product is obtained.

7.2.7 Detection of RT-PCR products

7.2.7.1 Preparation of samples

RT-PCR samples (20 μl) were added to 4 μl of gel loading buffer (Sigma) (supplied as a 6 times concentrate). Molecular weight (MW) markers ∅X174 Hae III (Sigma) (1.5 μl) were added to 2 μl of gel loading buffer (supplied at 6 times the working concentration) and 10 μl Millipore pure water. The molecular weight markers had band sizes of 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1353 base pairs.

7.2.7.2 Electrophoresis of samples

The RT-PCR products were run out on a 2% agarose gel (Pharmacia) containing 0.5 x TBE buffer (Gibco-BRL) (supplied at 10 times the working concentration: 1.0M Tris, 0.9M Boric Acid, 0.01M EDTA). A Jencon HU13 Midi Horizontal Electrophoresis Unit was used and the electrophoresis buffer was 1 x TBE. The gel was run at 150 V for 1 h 15 min and then stained for 10 – 15 min in an ethidium bromide solution containing 150 μl of a 1 mg ml^{-1} stock in 300 ml of distilled water.

The bands were visualised using a UV light box and photographs taken with a polaroid camera.

7.3 Results

7.3.1 DNase digestion of RNA preparations

This was done to remove all traces of contaminating DNA. DNA contamination was detected in the β-actin RT-PCR reactions when the samples were not DNase digested and no reverse transcriptase was added. DNA contamination could not be detected in either the TNF-α or IL-6 RT-PCR experiments regardless of whether or not the RNA preparations had been DNase treated since a band was only detected in tubes where reverse transcriptase had been added but not in its absence.

One problem which was encountered was that when the RNA preps were DNase digested and then purified, although RNA could be detected by spectrophotometry, the TNF-α and β-actin bands appeared very faint on the gel even with 3 μl of the appropriate RNA preparation added to the reaction mixture. In future studies, a greater number of cells could be stimulated to increase the starting concentration of RNA. This would result in a greater yield of RNA following DNase digestion and sample cleanup.

7.3.2 Optimisation of RT-PCR conditions

Various concentrations of RNA and cycle numbers were tested to try to reduce smearing of the bands in the gel. Tubes were set up for TNF-α, IL-6 and β-actin with different concentrations of RNA (2 μl) and PCR was carried out for 25 or 35 cycles. The optimal number of cycles was 25 for β-actin with an RNA concentration

of 75 ng added to the RT reaction. For IL-6, an RNA concentration of 176 ng added to the RT reaction and 25 cycles was optimal. For TNF-α an RNA concentration of 80 ng added to the RT reaction and 35 cycles was optimal.

To also reduce band smearing, a higher magnesium concentration was tried for both RT and PCR (5 μl of $MgCl_2$ solution added instead of 4 μl); however, the concentration recommended by the RT-PCR kit (Perkin-Elmer) with the appropriate concentration of RNA and cycle number was found to be the most successful.

7.3.3 Time course of expression for TNF-α and IL-6

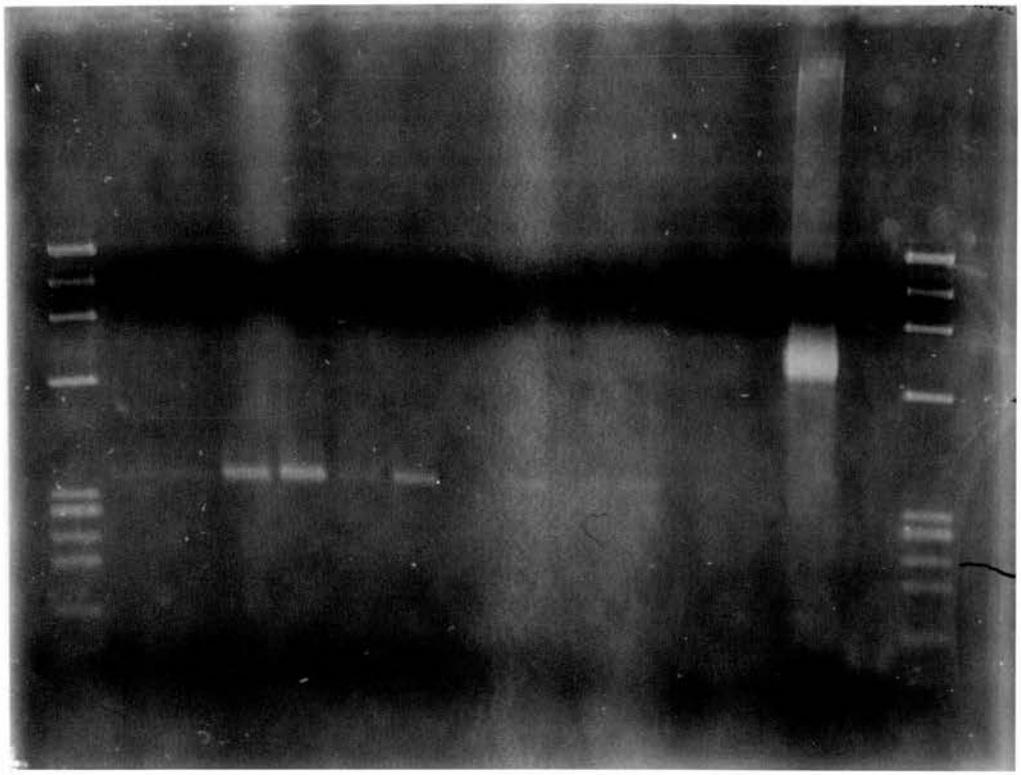
RNA extractions were carried out on cells to which no toxin had been added (negative controls) and on cells to which toxin was added. The cells were collected and frozen at -70°C at the following times: 0, 3, 6, 9, 12, 16, 20 and 24 h. The presence of both TNF-α and IL-6 mRNA was detected at 3 and 6 h only. The results are shown in Figures 7.2a and b and 7.3a and b. Gene expression was detected before maximum cytokine production in the supernatant was observed (6-9 h for TNF-α and 16 h for IL-6) and this was expected since peaks in gene expression are observed before peaks in production.

7.3.4 Non-specific stimulation of RNA production

There was some expression of TNF-α and IL-6 mRNA in cells which had not been incubated with toxin. This was more evident when foetal calf serum had been used in the assay system but could be reduced when homologous serum from the cell donor (Figure 7.4) or medium alone was used. When medium alone was used in the model system TNF-α but no IL-6 could be detected in the supernatant. At the level of gene expression, TNF-α but no IL-6 mRNA could be detected.

Figure 7.2

a) Time course of TNF-α mRNA production. Lane 1) MW markers ∅X174 Hae III; 2) time 0 h no TSST-1; 3) time 0 h plus TSST-1; 4) time 3 h no TSST-1; 5) time 3 h plus TSST-1; 6) time 6 h no TSST-1; 7) time 6 h plus TSST-1; 8) time 9 h no TSST-1; 9) time 9 h plus TSST-1; 10) time 12 h no TSST-1; 11) time 12 h plus TSST-1; 12) time 24 h no TSST-1; 13) time 24 h plus TSST-1; 14) β-actin positive control; 15) negative control; 16) MW markers ∅X174 Hae III



b) Time course of TNF-α detection in cell supernatant

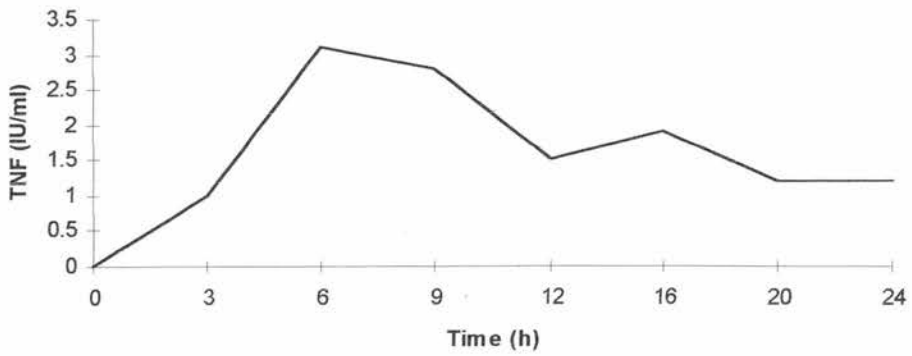
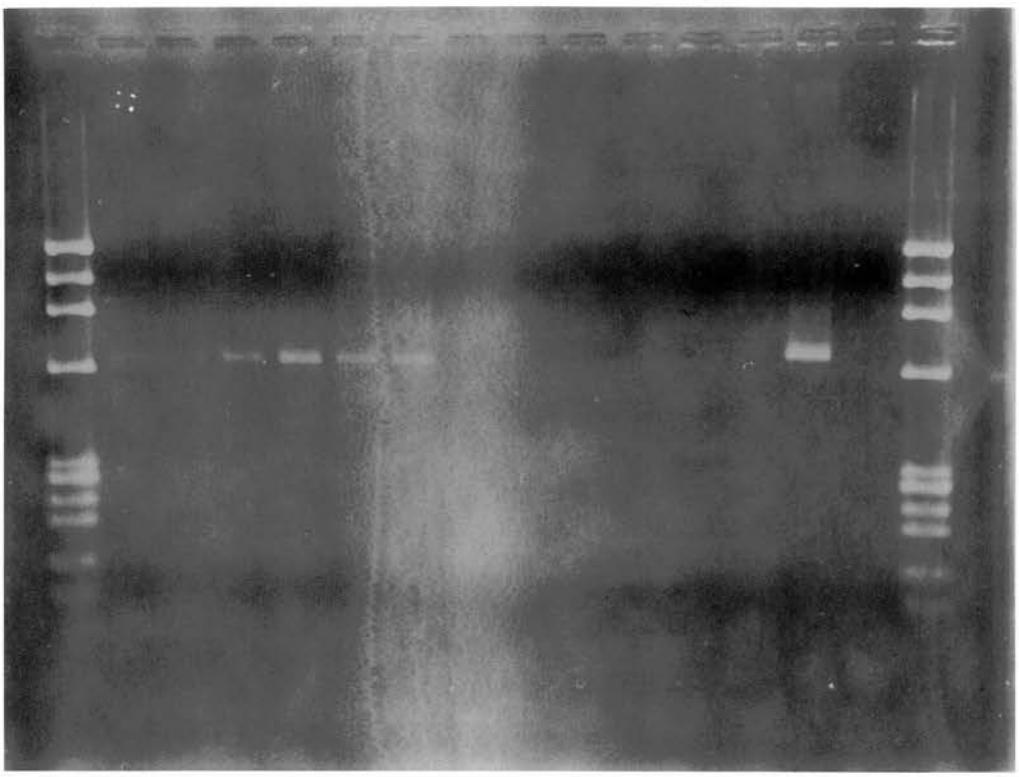


Figure 7.3

a) Time course of IL-6 mRNA production. Lane 1) MW markers ∅X174 Hae III; 2) time 0 h no TSST-1; 3) time 0 h plus TSST-1; 4) time 3 h no TSST-1; 5) time 3 h plus TSST-1; 6) time 6 h no TSST-1; 7) time 6 h plus TSST-1; 8) time 9 h no TSST-1; 9) time 9 h plus TSST-1; 10) time 12 h no TSST-1; 11) time 12 h plus TSST-1; 12) time 24 h no TSST-1; 13) time 24 h plus TSST-1; 14) β-actin positive control; 15) negative control; 16) MW markers ∅X174 Hae III



b) Time course of IL-6 detection in cell supernatant

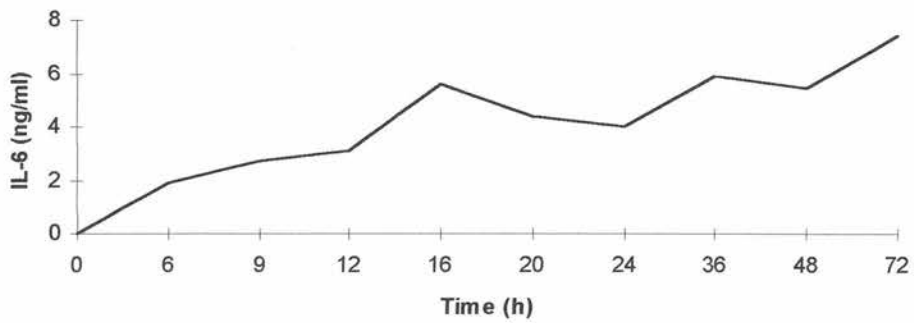
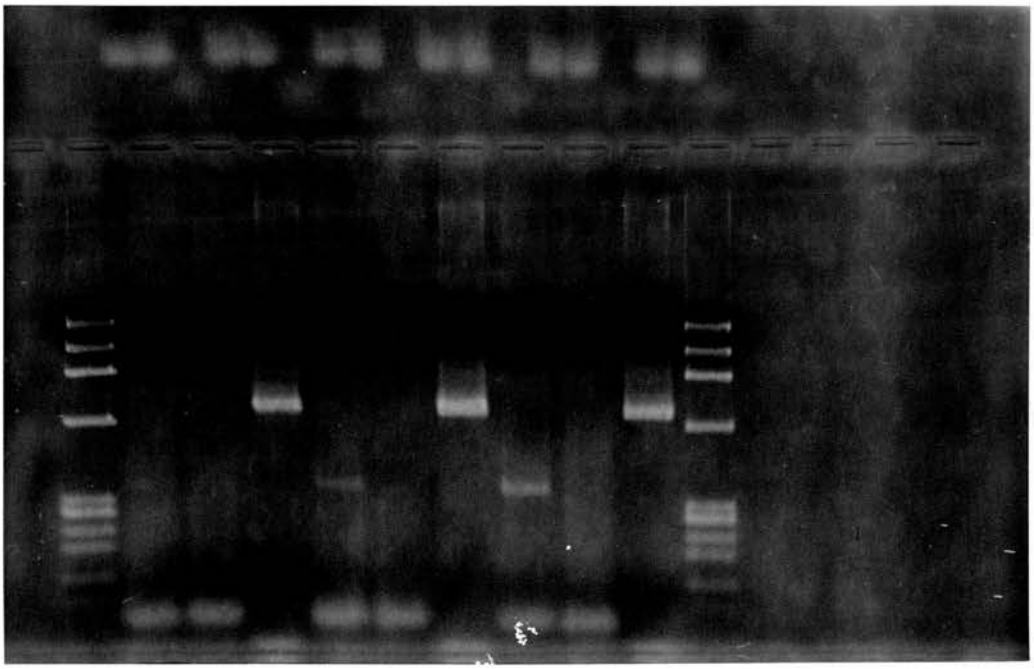


Figure 7.4 RT-PCR on cells using donor serum Lane 1) MW markers ∅X174 Hae III; 2) time 0 h, TNF-α plus RT; 3) time 0 h, TNF-α minus RT; 4) time 0 h, β-actin; 5) time 6 h no TSST-1, TNF-α plus RT; 6) time 6 h no TSST-1, TNF-α minus RT; 7) time 6 h no TSST-1, β-actin; 8) time 6 h plus TSST-1, TNF-α plus RT; 9) time 6 h plus TSST-1, TNF-α minus RT; 10) time 6 h, plus TSST-1 β-actin; 11) MW markers ∅X174 Hae III



7.3.5 Effect of freezing and formalin fixation on detection of TNF-α and IL-6 mRNA

7.3.5.1 Effect of freezing on mRNA detection

RNA extraction was carried out on cells which had been stored at -70°C for one year and two months and on cells which had been stored for 4 months. To overcome the problem of faint band detection after DNase digestion and clean up, and to ensure that any TNF-α band being detected was due to the presence of mRNA and not contaminating DNA, three different RT-PCR reactions were carried out for each sample: TNF-α primers plus reverse transcriptase added to the RT mixture; TNF-α primers minus reverse transcriptase added to the RT mixture; β-actin primers plus reverse transcriptase added to the mixture. To the TNF-α tubes, 3 μl of the appropriate RNA preparation were added whilst to the β-actin reactions, 2 μl of RNA and 1 μl of DEPC-treated water were added. Bands were detected for TNF-α in the tubes where RT had been added for both sets of cells but not in the tubes where no RT had been added. This indicates that the band was produced due to the presence of TNF-α mRNA and not due to any contaminating DNA. For β-actin, bands were detected for both sets of cells. The results are shown in Figure 7.5.

7.3.5.2 Effect of formalin on mRNA detection

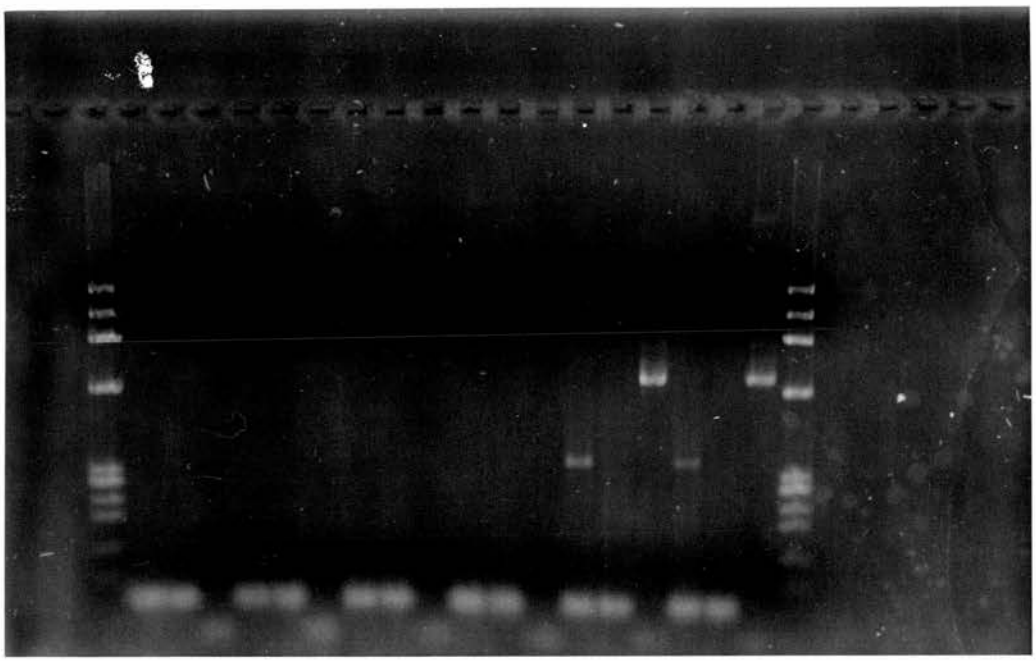
Cells which had been stimulated with TSST-1 and incubated for 3 or for 6 h were pelleted by centrifugation, the freezing medium removed and 500 μl of 0.1% or 4% buffered formalin (pH 6.7-7.33 (BDH) prepared in sterile PBS (pH 7.2)) added. Half the samples were left at room temperature whilst the other half were stored at 4°C.

Cells were stored under these conditions for 2 or 4 weeks after which RNA extraction was carried out.

Cells which had been stored for 2 weeks in 0.1% and 4% formalin at room temperature and at 4°C were used for RT-PCR. For each cell sample, three different RT-PCR reactions were carried out as described in 7.3.4.1. No TNF-α or β-actin bands were detected for any condition tested. The results are shown in Figure 7.5. Upon RNA quantitation by UV spectrophotometry, either no RNA or very low levels (less than one quarter of that detected in the frozen samples) could be detected.

From these results, it was concluded that using the developed method, gene expression could only be detected in frozen cells but not in cells which had been stored in formalin.

Figure 7.5 The effect of freezing and formalin on mRNA detection. Lane 1) MW markers ∅X174 Hae III; lanes 2 – 4, cells stored in 0.1% formalin at 4°C, TNF-α plus reverse transcriptase, TNF-α minus reverse transcriptase and β-actin respectively; lanes 5 – 7, cells stored in 0.1% formalin at room temperature, TNF-α plus reverse transcriptase, TNF-α minus reverse transcriptase and β-actin respectively; lanes 8 – 10, cells stored in 4.0% formalin at 4°C, TNF-α plus reverse transcriptase, TNF-α minus reverse transcriptase and β-actin respectively; lanes 11 – 13, cells stored in 4.0% formalin at room temperature, TNF-α plus reverse transcriptase, TNF-α minus reverse transcriptase and β-actin respectively; lanes 14 – 16, cells stored at -70°C for 4 months, TNF-α plus reverse transcriptase, TNF-α minus reverse transcriptase and β-actin respectively; lanes 17 – 19, cells stored at -70°C for one year and 2 months, TNF-α plus reverse transcriptase, TNF-α minus reverse transcriptase and β-actin respectively; 20) MW markers ∅X174 Hae III



7.4 Discussion

The experiments carried out for this part of the project were preliminary studies to try to develop a method for detection of gene expression in the tissues of SIDS infants.

Initial experiments involved determining the optimum cycle number and concentrations of RNA to use. The RT-PCR method was found to be more sensitive for detection of TNF-α mRNA than for IL-6 mRNA. A time course of TNF-α and IL-6 gene expression was then carried out. Expression of mRNA for both cytokines was detected at 3 and 6 h.

A higher number of cells should be used in future experiments. This would increase the initial RNA yield and allow the preparation to be DNase digested to remove any contaminating DNA. This would remove the problem of a β-actin band being observed when the RNA preparations were not DNase digested and no RT was added to the RT reaction mixture. This problem was not observed for either TNF-α or IL-6. To overcome the problem of DNA contamination observed for β-actin, in the preliminary studies, tubes were set up for TNF-α in the presence and absence of reverse transcriptase so that if a band was detected in the plus RT tube but not in the minus RT tube, it was due to the presence of mRNA for the TNF-α gene and not due to the presence of any contaminating DNA.

Foetal calf serum gave much higher levels of background stimulation of mRNA for both cytokines than either donor serum or medium alone. From Chapter 6, TNF-α but not IL-6 production was detected in supernatants from cells in which the assay medium was medium alone. RNA extraction was carried out on these cells and only TNF-α expression could be detected. For future *in vitro* studies, donor serum should be used in the assay system if both TNF-α and IL-6 gene expression are to be detected and this should help to keep non-specific background to a minimum.

TNF-α RNA could still be detected in cells which had been stored at -70°C for more than one year. No RNA was detected in cells which had been stored in either 0.1% or 4% formalin at room temperature or at 4°C.

In conclusion, these preliminary studies have developed a RT-PCR system to detect TNF-α and IL-6 gene expression. A number of problems were encountered and have been described. These would have to be taken in to consideration if this work was to be extended to tissue samples. RNA could still be detected in cells stored at -70°C but not in cells stored in formalin. In relation to tissues from SIDS infants, it can be suggested that either fresh or frozen tissue would need to be used to assess the presence of any cytokine mRNA. The detection of gene expression even in these tissues might depend on the interval between death and autopsy and the amount of tissue available for extraction. The studies were carried out with leukocytes in culture. Tissues such as lymph node or spleen might contain more cells that had been stimulated.

Chapter Eight

Discussion

8.1 Results of the study in relation to the original objectives

The role of breast feeding in protecting infants against SIDS has been suggested to be related to nutritional requirements for brain development [Williams, 1994; Schulze *et al.*, 1995]. In this study, breast feeding was examined in relation to the hypothesis that some SIDS deaths are due to uncontrolled inflammatory responses in response to infectious agents and / or cigarette smoke during a time in which infants are less able to control their inflammatory responses [Blackwell *et al.*, 1995a].

The specific objectives of the study were to address the following questions:

1) Do the methods used in most binding studies reflect the interactions at the mucosal surface?
2) What were the effects of human milk and infant formula on binding of a respiratory and gastrointestinal pathogen implicated in SIDS to epithelial cells?
3) What were the effects of oligosaccharide components on bacterial binding to epithelial cells?
4) Are there antibodies to the toxins implicated in SIDS present in human milk?
5) Can components in human milk reduce the ability of the staphylococcal toxins to induce inflammatory mediators?
6) Are the night-time levels of cortisol sufficient to control inflammatory responses to staphylococcal toxins?

8.1.1 Effect of experimental method on bacterial binding studies

The hypothesis proposed in this section was that breast milk, compared to infant formula, is better able to reduce the density of colonisation of epithelial cells by *S. aureus* and *C. perfringens*. This section had two aims: 1) to compare the ability of human milk and infant formula for their effect on binding of *S. aureus* and *C. perfringens* to epithelial cells; 2) to assess the effects of synthetic Lewis^a or Lewis^b antigens on bacterial binding.

Two different methods were used in the bacterial binding studies. Most binding studies employ the conventional experimental or *in vitro* approach in which the bacteria are treated with the potential inhibitor before they are added to target cells. The *in vitro* method is more useful for identifying components involved in binding and inhibition of binding. The *in vivo* approach was developed to try to simulate more closely the competitive interactions between cells, bacteria, human milk, infant formula or Lewis antigens that occur on mucosal surfaces.

For studies involving breast milk and infant formula, significantly different results between the two methods were obtained for both *S. aureus* and *C. perfringens*. With the *in vivo* method, breast milk enhanced bacterial binding to cells whilst infant formula inhibited binding. In contrast with the *in vitro* method, both breast milk and infant formula caused enhanced bacterial binding to cells. Enhancement of binding was found to be due to bacterial aggregation and greater enhancement of binding

was observed for the *in vitro* method because the bacteria had longer to aggregate before addition to the epithelial cells.

8.1.2 The effects of human milk and infant formula preparations on bacterial binding

The results obtained for the binding studies involving human milk and infant formula were the opposite of the original hypothesis. For both *S. aureus* and *C. perfringens*, regardless of method, breast milk caused bacterial aggregation and enhanced binding. Based on these results, it can be suggested that protection associated with breast feeding in relation to SIDS could be due partly to enhanced clearance of bacterial aggregates. Bacterial aggregates would be more readily engulfed by phagocytes than single bacteria resulting in greater bacterial clearance.

A second mechanism for protection might be inhibition of binding by free oligosaccharides. The presence of higher quantities of Lewis antigens in breast milk compared to infant formula might also act to reduce binding. This was examined in the next set of experiments.

8.1.3 The effects of oligosaccharides in human milk on bacterial binding

The Lewis^a antigen has been found to be a receptor for *S. aureus* and expression of the antigen parallels the peak in isolation of *S. aureus* from infants in the 2 - 4 month age range. Human milk has a unique content of free oligosaccharides and glycoconjugates that act as analogues of epithelial cell receptors. These can bind

bacterial toxins or adhesins and reduce the ability of bacteria to colonise epithelial surfaces [Andersson *et al.*, 1986; Goldman, 1993].

The synthetic Lewis^a and Lewis^b antigens inhibited binding of both *S. aureus* and *C. perfringens* in a dose dependent manner. This was observed with both the *in vivo* and *in vitro* methods. Lower antigen concentrations were required to cause maximum inhibition of binding using the *in vitro* method. This is because in the *in vitro* method there is direct blocking of the bacterial adhesins whilst with the *in vivo* method, the bacteria can either bind to the Lewis antigens on the cell's surface or to the Lewis antigens in solution.

Breast milk contains much higher levels of Lewis antigens compared to infant formula or cows' milk. The next set of studies investigated the hypothesis that the inhibitory activity of infant formula observed with the in vivo method might be increased by addition of Lewis antigens to formula. Although inhibition of binding was increased upon addition of Lewis antigens to formula, the decrease in binding was not significant. It might be that higher concentrations of Lewis antigens would need to be added to infant formula before a significant difference in inhibition of binding was observed.

8.1.4 The presence of antibodies in human milk, infant formula and cows' milk

Bacterial toxins produced on mucosal surfaces have been proposed to precipitate the series of events leading to a SIDS death. A second protective mechanism of breast

feeding might be the presence in breast milk, but not in infant formula, of antibodies against bacterial toxins implicated in SIDS.

Human IgA antibodies against the staphylococcal toxins TSST-1 and SEC and *C. perfringens* enterotoxin A were detected in breast milk but not in infant formula. Different human milk samples contained different antibody levels. These different levels of protective antibodies might confer different degrees of protection to an infant against bacterial toxins.

Antibody levels against TSST-1 and SEC were compared with information regarding isolation of *S. aureus* from the mother's nose or throat. *S. aureus* was isolated from around two thirds of the women whose milk samples had levels of IgA against TSST-1 or SEC greater than or equal to the mean; however, this difference was not significant compared to the isolation rate from women whose milk samples were below the mean. Some samples were found to have low levels of specific antibody to TSST-1 and / or SEC and had been taken when *S. aureus* was isolated from the mother. Possible explanations for this observation are that *S. aureus* isolated from these mothers did not produce the relevant toxins or that the mothers were recently colonised and had not yet developed IgA to the toxins.

IgG_1 is the main immunoglobulin involved in the mucosal immune system of ruminants [Lascelles *et al.*, 1986], and IgG_1 that bound TSST-1, SEC and CEA was detected in cows' milk but not in any of the infant formula preparations, or human

milk samples tested (the negative controls). This implies that formula fed infants receive no protective mucosal antibodies either human or bovine until they produce their own secretory IgA or they are given fresh cows' milk.

8.1.5 Neutralisation of TSST-1's activities by human milk or the synthetic Lewis antigens

Studies with breast milk or infant formula could not be carried out due to non-specific stimulation of cells treated with breast milk or formula in the absence of toxin. Some of the staphylococcal toxins bind to Lewis antigens on monocytes [Essery *et al.*, 1994; Essery, 1997]. Since Lewis^a and Lewis^b antigens are present in both human milk and infant formula, the model was used to test their effects on TNF-α and IL-6 production in response to TSST-1. No consistent effect on TNF-α production in response to TSST-1 was found for any concentration of Lewis^a or Lewis^b antigen tested. For IL-6, at both 6 and 24 h, a significant decrease in IL-6 production in response to TSST-1 was observed for all concentrations of Lewis^a tested. A significant decrease in IL-6 production was observed for Lewis^b (5 μg ml^{-1}) but at 24 h, a significantly increased response to TSST-1 and Lewis^b (10 μg ml^{-1}) was observed. This was not expected. The consistent results obtained with Lewis^a but not with Lewis^b might reflect the fact that all but about 3 - 5% of individuals express this antigen. Non-secretors usually have high levels of Lewis^a but secretors can also express high levels of Lewis^a. It might be that TSST-1 induces production of TNF-α and IL-6 by different pathways and this might explain the effect of Lewis^a on IL-6 production but not on TNF-α production. Further evidence for this

alternative pathway is provided by the fact that IL-6 production in response to TSST-1 can only be detected in the presence of serum (either FCS or donor). A recent study found that microbial superantigens stimulate T cells by cross-linking the MHC II molecule on accessory cells with the Vβ region of the T cell receptor; however, some superantigens including TSST-1 have been found to stimulate T cells by a cytokine pathway. The study found that production of IL-1 and IL-6 were induced in monocytes by all superantigens whereas TNF-α was induced in T cells and by some superantigens, also in monocytes [Rink *et al.*, 1997].

8.1.6 Night-time prevalence of SIDS and cortisol levels

Most SIDS deaths occur during the early hours of the morning and there is a significant decrease in night-time cortisol levels the week following the switch to an adult-like night-time temperature pattern. These observations are highly relevant to the hypothesis that inflammatory responses triggered by infection are involved in events which lead to a SIDS death since lower levels of the anti-inflammatory agent cortisol means that infants are less able to control their inflammatory responses.

In the model system, concentrations of cortisol (0.5 - 5 μg dl^{-1}) which were intended to represent those observed in infants at night after the development of circadian rhythm were insufficient to control inflammatory responses (TNF-α and IL-6) induced by TSST-1. Concentrations of cortisol greater then 5 μg dl^{-1} which were intended to represent concentrations observed in infants during the day and at night

prior to the change significantly decreased TNF-α and IL-6 production at 12 h and at 12 and 16 h respectively.

8.1.7 RT-PCR to detect gene expression

These were preliminary studies to try to devise methods to detect gene expression in the tissues of SIDS infants. Samples often arrive too late to detect cytokine production in the blood, especially if the cytokine is unstable. For example, IL-6 is stable and could be detected whilst TNF-α is often very difficult to detect.

Messenger RNA transcripts for TNF-α and IL-6 were detected at 3 and 6 h only. The RT-PCR methods were much more sensitive for detection of TNF-α mRNA compared with IL-6. To improve the method, the numbers of cells used could be increased. This would increase the final yield of RNA. Bands could be detected in the β-actin control wells even when no reverse transcriptase had been added. To overcome this problem, all RNA preps should be DNase digested prior to use. An increased RNA concentration would allow DNase digestion and clean up using the Qiagen RNA isolation kit to be carried out and leave sufficient quantities of RNA for use in RT-PCR. Foetal calf serum was found to give high background stimulation of mRNA in the absence of toxin. This was reduced in cells where homologous donor serum or medium alone had been used in the assay although the presence of medium alone was only suitable for detecting TNF-α gene expression.

This section also investigated the effects of freezing (stored at -70°C) and formalin fixation (0.1% and 4%) on mRNA detection since tissues from most SIDS infants have been stored in formalin. Cells were stored in 0.1% and 4% buffered formalin for 2 and 4 weeks at 4°C and at room temperature and then an RNA extraction carried out. No TNF-α or β-actin bands were detected using the 2 week RNA preparations. Both TNF-α and β-actin bands were observed with cells stored for a short or longer period of time at -70°C. The method might be applied to examination of frozen tissues from SIDS infants and comparison groups. While staphylococcal toxins have been detected in both frozen and formalin fixed tissues [Zorgani *et al.*, 1999], only frozen tissues would be appropriate for studies for detection of both toxins and mRNA of inflammatory mediators.

8.2 Results in relation to other studies

8.2.1 Hypotheses proposed to explain the mechanism of a SIDS death

A number of different hypotheses have been proposed to explain the mechanism of a SIDS death (Table 1.2). There is an increasing body of evidence to support the hypothesis that SIDS has an underlying infectious cause which can result in a SIDS death due to uncontrolled inflammatory responses. The common bacterial toxin hypothesis was proposed by Morris *et al*; [1987] and both toxigenic bacteria and their toxins have been isolated from SIDS infants. Virus infection has been implicated in SIDS [Howatson, 1992] and this might increase an infant's temperature to the levels required for induction of superantigenic toxin production

[Molony *et al.*, 1999]. Hypoglycaemia has also been proposed as a hypothesis for SIDS. Such metabolic problems would be exacerbated during times of stress such as minor infections [Burchell *et al.*, 1992]. Prolonged sleep apnoea has been proposed as another mechanism of a SIDS death. Infections might result in increased production of IL-1 and it has been suggested that IL-1 can cause prolonged sleep apnoea [Guntheroth, 1989]. TNF production in response to toxins can induce a hyperthermic response which might contribute to very high temperatures observed in some SIDS deaths [Sunderland *et al.*, 1981]. TNF can cause cardiac arythmias and reduce both myocardial contractility and vascular smooth muscle tone [Stevens *et al.*, 1993].

A number of hypotheses that have now been disproved. These include anaphylaxis in response to cows' milk since no IgE response was detected in SIDS infants. There is not much evidence to support a role for common drugs, poisons, carbon monoxide or lead. It has also been found that vaccination, rather than increasing the risk of SIDS as originally thought, decreases the risk.

8.2.2 Epidemiology

A number of epidemiological and case-control studies have been carried out to investigate the relationship between breast feeding, bottle feeding and SIDS [Ford *et al.*, 1993; Gilbert *et al.*, 1995]. A major problem with these studies is that contradictory results have been obtained. In general, the studies have found that although breast fed infants still die from SIDS, breast feeding reduces an infant's

risk whilst bottle feeding increases an infant's risk of dying from SIDS [Bernshaw, 1991]. Different levels of IgA to the three toxins examined might reflect variability of these antibodies in human milk. The levels needed to afford complete or partial protection need to be addressed.

8.2.3 Problems associated with studies of infant feeding

A number of fundamental problems are associated with investigations of infant feeding [Gilbert *et al.*, 1995]. The first problem relates to defining the method of feeding. Should a breast fed infant be described as one who is exclusively breast fed or should it include infants who are partly breast and partly bottle fed? [Cunningham *et al.*, 1991]. Non-exclusive methods of feeding will reduce any actual differences between breast and bottle feeding. In the UK, exclusively breast fed infants are rare and it has been found that 45% of babies received bottles of formula or water during their first week of life. It is also unethical to assign infants randomly to formula fed or breast fed groups [Bauchner *et al.*, 1986].

A second problem which is evident in infant feeding studies is the variability of both nutritional and immunological factors among breast milks from different mothers [Williams, 1994].

Confounding or associated variables are a third problem encountered by studies of feeding method. Feeding method correlates with socioeconomic factors and socioeconomic factors must be taken into account when analysing data obtained

from epidemiological studies. Breast fed infants are more likely to come from middle or upper class homes and have non-smoking parents. Women in higher social classes were also found to breast feed for longer. In contrast, formula fed infants are more likely to come from deprived areas and have parents who smoke [Cunningham *et al.*, 1991; Williams, 1994; Harrison, 1998, Blackwell *et al.*, 1999]. These factors make it difficult to distinguish between the possibilities that the protective effect of breast feeding in relation to SIDS is due to actual factors present in breast milk or due to a reduction of other risk factors which are associated with lower socioeconomic groups. Some studies have found that when the results are adjusted for maternal smoking, pre-term gestation and parental unemployment, the association between the risk of SIDS and bottle feeding is greatly reduced [Gilbert *et al.*, 1995].

8.2.4 Infection, breast feeding, formula feeding and SIDS

Infections have been suggested to play a role in the mechanism of a SIDS death. Breast fed infants have increased protection against gastrointestinal and lower respiratory disease [Buescher, 1994; Pisacane *et al.*, 1994]; therefore, breast feeding might reduce the risk of SIDS by providing protection against infection. These effects hold even when confounding variables have been taken in to consideration, and they are particularly obvious in countries such as Brazil where formula fed infants have been found to be 14.2 times more likely to die from diarrhoeal disease compared to breast fed infants. In contrast, in industrialised countries such as the UK where infant formulas are hygenically prepared and nutritionally complete, the

health benefits of breast feeding compared to formula feeding have been questioned [Williams, 1994].

Maternal IgG antibody is responsible for the major protection of an infant against bacteria and viruses. IgG specific for toxins would be important defences for neutralisation of the toxins implicated in SIDS. Levels of maternal IgG fall to about one quarter of their original levels in infants about three months after birth and the infant is more vulnerable at this time to a challenge by infectious agents.

It is possible that breast fed infants receive protection by secretory IgA in breast milk [Bernshaw, 1991]. No immunoglobulins are present in the intestinal tracts of bottle fed infants until they produce their own secretory IgA [Davidson *et al.*, 1987] and these appear later than breast fed infants [Dallas *et al.*, 1998]. Secretory IgA is a specific defence mechanism for the breast fed baby and provides local immunological protection at mucosal surfaces against bacterial or viral infection or by preventing antigens crossing the epithelial barrier. It can be suggested that IgA antibodies in breast milk which can bind to and neutralise toxin activity [Rolfe *et al.*, 1995, Dallas *et al.*, 1998] might neutralise the effects of bacterial toxins on mucosal surfaces reducing the possibility of systemic effects. Premature infants are at a greater risk of dying from SIDS and they have lower levels of IgG in their first months of life compared to full-term infants [Morris *et al.*, 1987]. It is especially important that these premature infants are breast fed and receive IgA.

8.2.5 Virus infection, bacterial toxins, method of feeding and SIDS

Respiratory infections have been implicated in some SIDS deaths, and an infant's chance of developing such disease is increased by synergistic interactions between smoking and bottle feeding. In a longitudinal study of mothers and infants, smoking and formula feeding were found to be significantly higher among lower socioeconomic groups [Blackwell *et al.*, 1999].

Several respiratory viruses including respiratory syncitial virus (RSV), parainfluenza virus, rhinovirus, adenovirus and influenza A virus have been identified in SIDS infants. RSV infection is associated with minimal breast feeding [Ruuskanen *et al.*, 1993]. Experimental studies indicate that viral infections increase the susceptibility of the respiratory tract to bacterial colonisation by toxigenic bacteria implicated in SIDS and other respiratory pathogens [Raza *et al.*, 1993; Saadi *et al.*, 1993; Saadi *et al.*, 1996; El Ahmer *et al.*, 1999].

Evidence for a synergistic interaction between virus infection and bacterial toxins has been found in several studies. The effects of staphylococcal (α and γ) and diphtheria toxins could be enhanced in 5 day old ferrets infected with influenza virus. Since there was no effect on viral replication, it was concluded that it was the virus which was having an effect on the infection [Jakeman *et al.*, 1991]. Induction of an auto-amplification of cytokine production might be one possible interaction between virus infections and toxins. The permeability of cells can be enhanced by influenza virus infection and this might allow more or rapid uptake of the toxin. The

increased permeability will also allow increased release of inflammatory mediators [Jakeman *et al.*, 1991]. Subclinical infections with lymphocytic choriomeningitis virus was found to prime mice expressing the SEB Vβ receptor and resulted in induction of rapid fatal shock with sublethal concentrations of SEB [Sarawar *et al.*, 1994].

Antibodies and other components of human milk have neutralising effects on many respiratory viruses. In addition the anti-viral activity of human milk could be important for reducing the risk of SIDS.

8.2.6 Breast feeding and control of inflammatory responses

During the 2 – 4 month age range, infants become more reliant on their inflammatory responses since maternal antibody levels against bacteria and viruses are decreasing. Immunisation studies have shown that younger infants are better able to control their inflammatory responses compared to older infants.

Breast fed infants develop circadian rhythm patterns significantly earlier than formula fed infants. Breast fed infants will be able to neutralise a challenge from bacteria or their toxins due to the presence of maternally derived IgA in breast milk. Infants (*e.g.* Asian) who develop adult-like night-time temperature patterns and circadian rhythm patterns of cortisol levels later compared with normal infants, will also be able to control an inflammatory response to bacteria or their toxins since these infants will have begun to produce their own antibodies and will have

benefited from the extra time to produce active immunity through vaccination and natural exposure. Since a greater number of Asian infants are breast fed at least during the first 4 months of life [Lawson, 1998], they would appear to have two advantages in relation to protection against SIDS.

If the switch to the circadian rhythm pattern occurs in infants when maternal antibodies are low, the infant is formula fed or they have not yet begun to produce their own antibodies, the infant is at an increased risk of SIDS due to lower night-time cortisol levels which are unable to control the associated inflammatory responses.

8.3 Future work

8.3.1 Effects of the synthetic Lewis antigens on bacterial binding and toxin activity

The effect of adding higher concentrations of Lewis antigens to infant formula needs to be examined as these naturally occurring substances might improve protection of mucosal surfaces against some bacterial pathogens (*H. pylori*, *S. aureus*, *B. pertussis* and *C. perfringens*) that use these antigens as mucosal receptors.

To investigate the effects of Lewis antigens on IL-6 production, the experiments could be repeated in relation to a quantitative assessment of the level of Lewis antigens on the leukocytes. These experiments also need to be repeated using a slightly modified protocol in which TSST-1 is initially incubated with the appropriate concentration of Lewis antigen for 2 h at 37°C before addition to the cells. The effects of lower levels of both antigens need to be tested in these studies.

8.3.2 Cortisol levels

The studies by Wailoo and colleagues need to be extended to determine when an infant's night-time cortisol levels rise above 5 $\mu g\ dl^{-1}$ into a range that could control the inflammatory responses. The development of these physiological changes in ethnic groups (*e.g.* Native Americans, Australian Aborigines) in which there is a high incidence of SIDS also need to be examined.

8.3.3 Method of feeding and smoking

In populations in which breast feeding is not strongly associated with socioeconomic group, it has been found to be protective against the risk of SIDS and against enteric and respiratory tract infections. Bottle feeding is largely determined by social and cultural factors and is strongly associated with maternal smoking [Gilbert *et al.*, 1995]. To overcome some of these problems associated with investigation of SIDS in relation to method of feeding, studies need to be carried out in countries such as New Zealand where breast feeding is not associated with socioeconomic status to determine the effects of maternal smoking on density of colonisation by normal and pathogenic micro-organisms. It would also be important to investigate the effects of bottle and breast feeding on density of colonisation of infants of mothers who smoke and mothers who do not smoke.

8.3.4 Method of feeding and response to infection

Studies carried out by other groups on physiological responses to immunisation (temperature, night-time cortisol levels) [Westaway *et al.*, 1993] need to be assessed in relation to method of feeding to determine if inflammatory responses (temperature) are better controlled in breast fed infants.

8.3.5 Genetic control of inflammatory responses to bacterial toxins

Studies indicate that there are significant genetic factors associated with control of both pro-and anti-inflammatory responses to endotoxin [Westendorp *et al.*, 1995]. Fatal outcome of meningococcal infection was related to low levels of TNF

responses and / or high levels of IL-10 responses obtained with *in vitro* studies of first degree relatives of the patients [Westendorp *et al.*, 1997].

Future work on SIDS will compare the inflammatory responses to common bacterial toxins between parents who have had a SIDS death and the responses of parents who have not. The studies will also include comparison of inflammatory responses of parents from various ethnic groups to determine if some of the differences in the incidence of SIDS, particularly those of Asian infants, are due to genetic differences in their inflammatory responses or differences in their development noted by Petersen and Wailoo [Petersen *et al.*, 1994].

8.4 Conclusions

There is a growing body of evidence relevant to risk factors for SIDS that indicates microorganisms might precipitate the series of events leading to death of the infant. This study investigated the protective effect of breast feeding in relation to SIDS at two stages of the bacterial toxin model: 1) at the level of colonisation by toxigenic bacteria; 2) control of inflammatory mediators produced in response to bacterial toxins.

In bacterial binding studies using the *in vivo* method, breast milk enhanced bacterial binding to cells and this was found to be due to bacterial aggregation. In contrast, infant formula inhibited bacterial binding to cells and it has been suggested that one protective effect of breast feeding in relation to SIDS might be that in breast fed infants, aggregate formation leads to enhanced clearance of bacteria by phagocytosis, ciliary action or peristalsis. The results obtained for the binding studies disproved the original hypothesis proposed, that breast milk might be better than infant formula at inhibiting bacterial binding to epithelial cells; however, the results could be explained to suggest a protective effect of breast feeding in relation to SIDS. Reduction in bacterial binding observed with infant formula in the *in vivo* method could afford some protection by reducing colonisation by potentially pathogenic bacteria; however, if damage to the host is mediated by toxins, the formula preparations lack antibodies to the toxins and have very low levels of the Lewis antigens.

As expected, breast milk but not infant formula contained human IgA antibodies to the toxins TSST-1, SEC and CEA. These antibodies might also protect a breast fed infant by neutralising bacterial toxins on mucosal surfaces and preventing them entering the bloodstream or by binding to adhesins on pathogens and interfering with their attachment. This protection would be particularly important during the period when cortisol levels drop at night-time following development of circadian rhythm. IgG_1 antibodies against TSST-1, SEC and CEA were detected in cows' milk but not in infant formula preparations. This implies formula fed infants receive no protective antibodies either human or bovine until they begin to produce their own secretory IgA or they are fed fresh cows' milk.

If the switch to the circadian rhythm pattern occurs early when maternal antibodies are present, the infant is receiving IgA in breast milk or later when immunisation or natural exposure has induced protective antibodies, the infant might be able to reduce the effects of a challenge with bacterial toxins or infectious agents. If the switch occurs in an infant when maternal antibodies are low, the infant is formula fed or the infant has not produced its own active immunity, lower night-time cortisol levels might be insufficient to control inflammatory responses induced by bacterial toxins alone or in combination with other infections. These uncontrolled inflammatory responses might precipitate the series of events leading to SIDS.

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Appendix - List of Publications

Al Madani, O., Gordon, A.E., Weir, D.M., Raza, M.W., Busuttil, A. and Blackwell, C.C. (1999) Pyrogenic toxins of *Staphylococcus aureus* in sudden unexpected nocturnal deaths in adults and older children: factors influencing the control of inflammatory responses to toxic shock syndrome toxins. FEMS Immunology and Medical Microbiology (in press).

Gordon, A.E., Al Madani, O., Weir, D.M., Busuttil, A. and Blackwell, C.C. (1999) Cortisol levels and control of inflammatory responses to toxic shock syndrome toxin-1 (TSST-1): the prevalence of night-time deaths in sudden infant death syndrome (SIDS). FEMS Immunology and Medical Microbiology (in press).

Gordon, A.E., Saadi, A.T., MacKenzie, D.A.C., James, V.S., Elton, R.A., Weir, D.M., Busuttil, A. and Blackwell, C.C. (1999) The protective effect of breast feeding in relation to sudden infant death syndrome: II. The effect of human milk and infant formula preparations on binding of *Clostridium perfringens* to epithelial cells. FEMS Immunology and Medical Microbiology (in press).

Gordon, A.E., Saadi, A.T., MacKenzie, D.A.C., Molony, N., James, V.S., Weir, D.M., Busuttil, A. and Blackwell, C.C. (1999) The protective effect of breast feeding in relation to sudden infant death syndrome (SIDS): III. Detection of IgA antibodies in human milk that bind to bacterial toxins implicated in SIDS. FEMS Immunology and Medical Microbiology (in press).

Saadi, A.T., Gordon, A.E., MacKenzie, D.A.C., James, V.S., Elton, R.A., Weir, D.M., Busuttil, A. and Blackwell, C.C. (1999) The protective effect of breast feeding in relation to SIDS: I. The effect of human milk and infant formula preparations on binding of toxigenic *Staphylococcus aureus* to epithelial cells. FEMS Immunology and Medical Microbiology (in press).